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Title of Thesis: "Studies of a Bulbospinal Pathway that Regulates Cardiovascular Function: Inhibition by GABA at the Ventral Medulla and Mediation by Spinal Cord Substance P"

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ABSTRACT

Title of Dissertation: Studies of a sympathoexcitatory bulbospinal pathway that regulates cardiovascular function: inhibition by GABA at the ventral medulla and mediation by spinal cord substance P

Jill R. Keeler, Doctor of Philosophy, 1984

Dissertation directed by: Cinda J. Helke, Assistant Professor, Department of Pharmacology

The central nervous system plays a prominent role in regulating the cardiovascular system, and the ventral medulla (VM) specifically has been the focus of recent attention as a brain region important in cardiovascular control. Pharmacologic approaches were used to characterize the role of the VM and the mechanisms by which information is relayed to the heart and vasculature.

Initially, experiments were done to evaluate a rat model for studying the cardiovascular effects of pharmacologic manipulations of the VM. GABAergic drugs were used because of their well-characterized actions at the VM in other species. GABA and the GABA receptor agonist, muscimol, applied to a discrete region of the exposed surface of the VM, produced dose-dependent decreases in mean arterial pressure (MAP) and heart rate (HR) that were reversed with the GABA receptor antagonist, bicuculline. Bicuculline alone raised MAP and HR. The GABAergic drug-induced effects were blocked by sympathetic blockers injected intravenously. The most sensitive site was localized to an intermediate area on the surface of the VM. Topical application of [^{3}H]GABA to this intermediate area resulted in labeling that was concentrated at the site of application, measured quantitatively and autoradiographically, and most

closely corresponded to the lateral paragigantocellular nucleus. These data provided evidence for a neuronal system near the surface of the VM of the rat that increases sympathetic outflow to the cardiovascular system and is tonically inhibited by GABA.

Because substance P (SP) is contained in VM projections to the intermediolateral cell column (IML) of the spinal cord, and SP excites sympathetic preganglionic neurons when injected into the IML, the second series of experiments were done to determine if SP in the spinal cord was responsible for mediating the GABAergic effects. Anesthetized rats were given intrathecal (i.t.) injections of SP antagonists. Three SP antagonists (50 μg) decreased MAP to 2/3 baseline levels, but did not change HR. They also blocked the increases in MAP and HR evoked by application of bicuculline to the VM, and their action persisted for hours. A lower dose (5 μg) of a SP antagonist produced the same effects which were reversed in 1-2 hours. Intrathecal injections of a stable SP receptor agonist, [pGlu^5, MePhe^8, MeGly^9]-SP (DiME-SP), produced dose-dependent increases in MAP and HR which were accompanied by increases in plasma epinephrine and norepinephrine. Intravenous injection of a ganglionic blocker inhibited the cardiovascular and catecholamine responses to DiME-SP i.t. DiME-SP i.t. countered SP antagonist mediated 1) hypotensive responses and 2) antagonistic effects on bicuculline-induced sympathoexcitatory responses evoked from the VM.

In summary, these studies provide pharmacologic evidence that excitatory cardiovascular effects evoked by the stimulation of cell bodies in the VM are due largely to SP transmission in the spinal cord, and these effects are mediated by the sympathetic nervous system.

STUDIES OF A SYMPATHOEXCITATORY BULBOSPINAL PATHWAY THAT REGULATES CARDIOVASCULAR FUNCTION: INHIBITION BY GABA AT THE VENTRAL MEDULLA AND MEDIATION BY SPINAL CORD SUBSTANCE P

by

Jill R. Keeler

Dissertation submitted to the Faculty of the Department of Pharmacology
Graduate Program of the Uniformed Services University of the
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DEDICATION

I dedicate this tome to the three people who were dedicated to providing me with the stamina necessary to persevere for 4+ years in achieving this goal.

Curt and Pat Keeler, my parents: for their quarterly visits of encouragement, for being my best fans, and for their love.

Sharon Martin: for her loyal friendship, understanding, and comradery "behind the books, in the lab, and on the trail".

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To my advisory committee, Dr.'s Aronow, Barrett, Muldoon, and Sarvey: The body of this work was much enhanced because of your thoughtful and constructive criticisms...thank you.

To my cohabitants in the lab, Elaine Phillips, et al.: Thanks for being around so I could unload my frustrations and share my elations.

I owe special gratitude to the Army Nurse Corps, in particular COL Sarah Halliburton, COL James Hunn (ret.), and BG Hazel Johnson (ret.) for supporting the completion of my degree; and to the University and the Department of Pharmacology for going to bat for me.

Success as a graduate student is directly proportional to one's moral support systems. God bless mine, even in absentia. Sincere thanks go to Pigroasters and other comrades around the world for their faith and loyalty during my seclusion years.

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ABBREVIATIONS

A1, A5	norepinephrine containing cell groups
B3	serotonin containing cell group
BMI	bicuculline methiodide
C1, C2, C3	epinephrine containing cell groups
CNS	central nervous system
CSF	cerebrospinal fluid
DH	dorsal horn of the spinal cord
5,7-DHT	5,7-dihydroxytryptamine, a 5-HT neurotoxin
DiME-SP	[pGlu^{5}, MePhe^{8}, MeGly^{9}]-SP(5-11), a SP agonist
DMNX	dorsal motor nucleus of the vagus
GABA	γ-aminobutyric acid
HR	heart rate
HRP	horseradish peroxidase
5-HT	serotonin
IML	intermediolateral cell column of the spinal cord
i.t.	intrathecal
L area	caudal chemosensitive area of the VSMO
M area	rostral chemosensitive area of the VSMO
MAP	mean arterial pressure
NTS	nucleus of the solitary tract
PBS	phosphate buffered saline
PGCL	lateral paragigantocellular nucleus
PNMT	phenylethanolamine-N-methyltransferase
S area	intermediate chemosensitive area of the VSMO
SHR	spontaneously hypertensive rat
SPR	stroke prone rat
SP	substance P
SP antagonist I	[D-Pro^{2}, D-Trp^{7,9}]-SP
SP antagonist II	[D-Pro^{2}, D-Phe^{7}, D-Trp^{9}]-SP
SP antagonist III	[D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}]-SP
SP antagonist IV	[D-Pro^{4}, D-Trp^{7,9,10}]-SP(4-11)
VH	ventral horn of the spinal cord
VSMO	ventral surface of the medulla oblongata
WKY	Wistar-Kyoto rat

INTRODUCTION

The central nervous system (CNS) plays a prominent role in the regulation of the cardiovascular system. The cell bodies of preganglionic sympathetic and parasympathetic neurons are located in the CNS and these neurons influence the function of virtually every vascular bed as well as the heart. Neurons in the CNS exert tonic excitatory and inhibitory influences on autonomic nerves to maintain the integrity of the cardiovascular system. Sensory information is relayed to the CNS where it is assimilated, then reflex responses are initiated to maintain the appropriate physiologic state of the cardiovascular system. The CNS also regulates cardiovascular function indirectly by its influence over the release of vasoactive hormones [reviews by Antonaccio, 1984; Baum, 1984]. Cardiovascular related pathologies of suggested neural etiology such as certain types of hypertension [Kedzi, 1967; Korner, 1970; Reis and Doba, 1974; Esler et al., 1977; DeFeudis, 1981; Abboud, 1982, 1984], cardiac dysrhythmias [Abildskov, 1975], orthostatic hypotension [Johnson, 1983; Kuroiwa et al., 1983], vasospastic angina [Toyama et al., 1979; Graham et al., 1983], and atherosclerosis [DeFeudis, 1981] further support the importance of the CNS in cardiovascular regulation.

Within the CNS are highly complex neuronal networks where specific neurotransmitters and neuromodulators serve to communicate information that ultimately results in cardiovascular phenomena. Knowledge of these chemical mediators as well as their metabolic mechanisms and receptive elements are crucial to the understanding of normal and abnormal CNS control of the cardiovascular system and sites and mechanisms of drug actions that alter cardiovascular function.

Areas in the CNS believed to be involved with cardiovascular

control include portions of virtually every level of the neuraxis [Korner, 1979; Galosy et al., 1981; Loewy, 1982]. One area, at or near the ventral surface of the medulla oblongata (VSMO), has been the focus of recent attention as a brain region important in cardiovascular control.

Dittmar [1870, 1873] was the first to report that there was a "vasomotor center" in the ventral medulla. While monitoring blood pressure in curarized rabbits, he made successive rostral to caudal knife cuts in the brainstem, and noted a precipitous drop in blood pressure when the brainstem was transected in the rostral medulla (at the level of the facial nuclei). Blood pressure was maintained, however, when the dorsal 2/3 of the medulla was destroyed at the same rostrocaudal level and Dittmar concluded that the site of the vasomotor center was in the ventral reticular formation near the facial nuclei. These potentially important findings laid dormant for a century.

It was an investigation of sites of drug action that first inspired the current interest in the ventral medulla's cardiovascular role. Feldberg and Guertzenstein [1972] reported that injection of pentobarbital into the lateral cerebroventricle of a cat decreased the blood pressure. Experiments in which the drug was restricted to discrete areas of the ventricular system showed that the site of action was the VSMO. Indeed, results of subsequent studies showed that the ventral medulla was selectively sensitive to a variety of pharmacologic agents which produced alterations in cardiovascular function, and are listed in Table 1.

In addition to cardiovascular phenomena, changes in ventilation [Mitchell et al., 1963; Cozine and Ngai, 1967; Dev and Loeschcke,

Table 1

AGENTS THAT INDUCE CARDIOVASCULAR CHANGES WHEN TOPICALLY APPLIED TO OR MICROINJECTED NEAR THE VSMO

acetylcholine	Dev and Loeschcke, 1979a,b
γ-aminobutyric acid	Guertzenstein, 1973; Feldberg, 1976; Wennergren and Oberg, 1980; Feldberg and Rocha e Silva, 1981; Yamada et al., 1982; Blessing and Reis, 1982; Willette et al., 1983b; Keeler and Helke, 1984; Keeler et al., 1984a; Ross et al., 1984a
ammonium chloride	Loeschcke, 1980
atropine	Guertzenstein, 1973; Feldberg and Guertzenstein, 1976; Dev and Loeschcke, 1979b; Wennergren and Oberg, 1980
bicuculline	Feldberg, 1976; Feldberg and Rocha e Silva, 1978; Williford et al., 1981; Yamada et al., 1982, 1983, 1984; Blessing and Reis, 1983; Willette et al., 1983b; Keeler and Helke, 1984; Keeler et al., 1984a,b; Ross et al., 1984a
carbachol	Guertzenstein, 1973; Feldberg and Guertzenstein, 1976
clonidine	Bousquet and Guertzenstein, 1973; Bousquet et al., 1975
cyanide	Loeschcke, 1980
dyflos	Edery and Guertzenstein, 1974
epinephrine	Dev and Loeschcke, 1979b
glutamate	Dampney, 1981; Dampney et al., 1982; Blessing and Reis, 1982; Ross et al., 1983, 1984a; Willette et al., 1983a,b
glutamate diethylester	Willette et al., 1983a
glycine	Guertzenstein, 1973; Guertzenstein and Silver, 1974; Feldberg and Guertzenstein 1976; Guertzenstein et al., 1978; Wennergren and Oberg, 1980; Feldberg and Roche e Silva, 1981; Blessing and Reis, 1983; Keeler et al., 1984a
hexamethonium	Feldberg, 1976; Feldberg and Guertzenstein, 1976

Table 1 (continuation)

kainic acid	Blessing et al., 1981b; Loewy and Sawyer, 1982; McAllen et al., 1982; Ross et al., 1984a
leptazol	Guertzenstein, 1973; Feldberg and Rocha e Silva, 1978; Guertzenstein and Lopes, 1984
lobeline	Loeschcke, 1980
met-enkephalin	Florez and Mediavilla, 1977
methyldopa	Minson et al., 1984
morphine	Hurle et al., 1982
muscimol	Bousquet et al., 1981a; Williford et al., 1981; Yamada et al., 1982; Blessing and Reis, 1983; Willette et al., 1983a,b; Keeler et al., 1984a
naloxone	Hurle et al., 1982
nicotine	Armitage and Hall, 1967; Feldberg and Guertzenstein, 1976; Feldberg and Rocha e Silva, 1978; Dev and Loeschcke, 1979a,b; Guertzenstein and Lopes, 1984
norepinephrine	Dev and Loeschcke, 1979b
pentobarbital	Feldberg and Guertzenstein, 1972; Hurle, et al., 1982; Yamada et al., 1983; Guertzenstein and Lopes, 1984
physostigmine	Guertzenstein, 1973; Feldberg and Guertzenstein, 1976; Wennergren and Oberg, 1980
picrotoxin(in)	Feldberg, 1976; Feldberg and Rocha e Silva, 1978; Yamada et al., 1984
procaine	Dev and Loeschcke, 1979a
strychnine	Guertzenstein, 1973; Feldberg and Rocha e Silva, 1978; Blessing and Reis, 1983; Keeler et al., 1984a
tubocurarine	Guertzenstein, 1973; Feldberg and Rocha e Silva, 1978
tetrodotoxin	Ross et al, 1983, 1984a
veratridine	Loeschcke, 1980

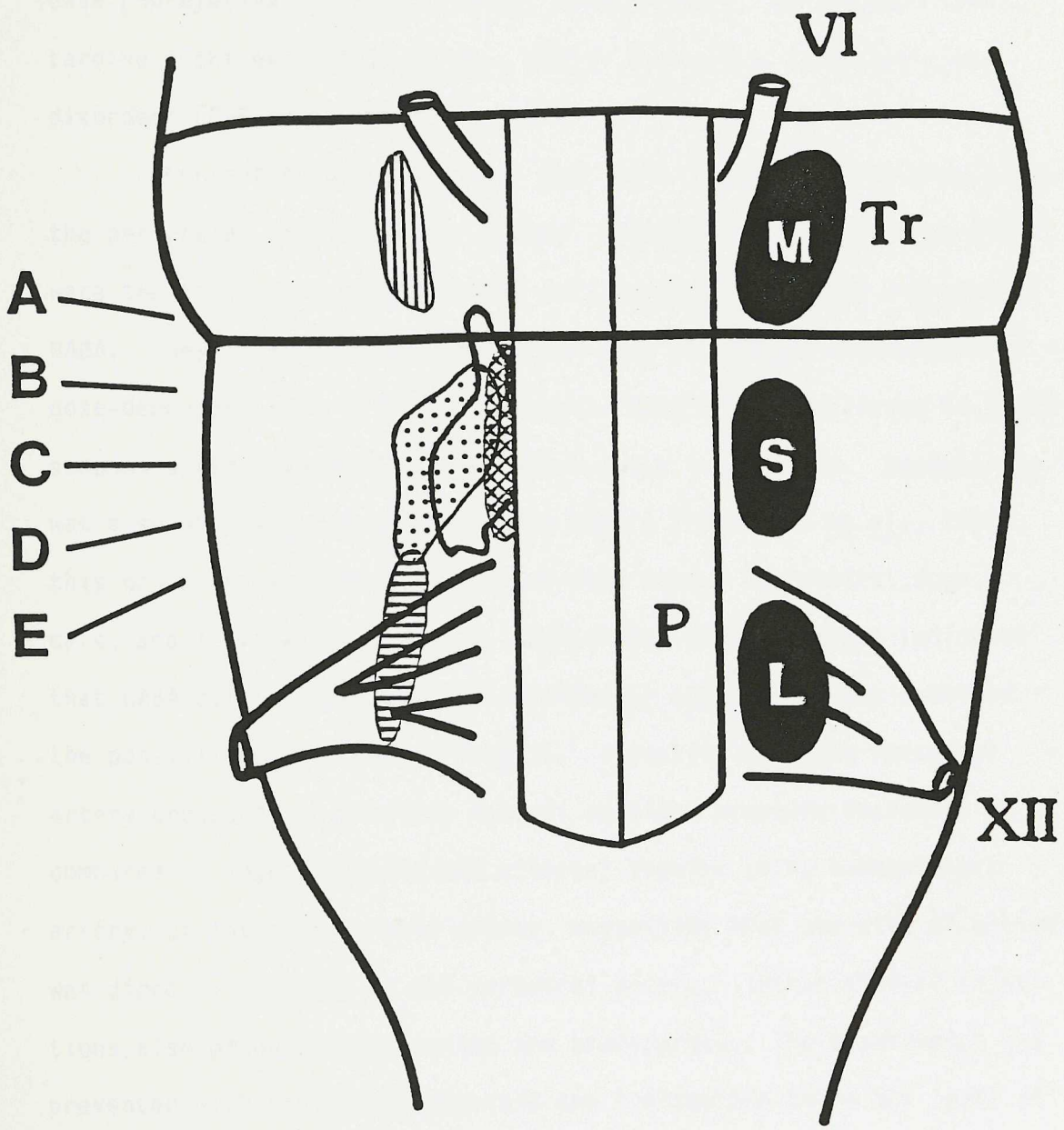
1979a,b; Schlaefke and See, 1980; Malcolm et al., 1980; Schlaefke, 1981; Florez et al., 1982; Loeschcke, 1982; Yamada et al., 1982, 1983; Wennergren and Wennergren, 1983], blood glucose [Dey et al., 1975; Feldberg, 1976], vasopressin levels [Bisset et al., 1975; Feldberg, 1976; Feldberg and Rocha e Silva, 1978, 1981; Ross et al., 1984a], and nociception [Dey and Feldberg, 1976; Feldberg, 1976; Akaike et al., 1978; Takagi et al., 1978; Watkins et al., 1983] due to drugs acting in this region have also been reported.

Based on the cardiovascular and ventilatory responses to drugs, three distinct areas bilateral to the pyramids have been recognized and defined in the cat (Figure 1): a rostral area over the trapezoid body (area M) [Mitchell et al., 1963], a caudal area where the hypoglossal rootlets emerge from the medulla (area L) [Loeschcke and Koepchen, 1958], and an intermediate area between these (area S) [Schlaefke and Loeschcke, 1967]. It is the intermediate zone from which the most profound cardiovascular effects have been evoked [Feldberg and Guertzenstein, 1972; Guertzenstein and Silver, 1974; Feldberg, 1976; Feldberg and Guertzenstein, 1976; Feldberg and Rocha e Silva, 1978; McAllen et al., 1982; Yamada et al., 1982; Hurle et al., 1982].

Most of these studies on the sensitivity of the VSMO to drugs were done in cats, and of these drugs, γ-aminobutyric acid (GABA) has been investigated most thoroughly. Actually, GABA was implicated as a cardiovascular modulator in the CNS long before the VSMO was implicated as a probable site of action for the cardiovascular effects produced by this neurotransmitter.

GABA is now widely accepted as an important inhibitory transmitter in the CNS [review by Krnjević, 1976; Curtis, 1979; Cooper

Figure 1. Schematic diagram of the ventral surface of the medulla oblongata (VSMO) of the rat and underlying structures. Left: lateral paragigantocellular nucleus (bold outline), A5 cell group (vertical lines), C1 cell group (dotted), lateral extension of B3 cell group (cross-hatched), A1 cell group (horizontal lines). Right: surface chemosensitive zones M, S, L. P (pyramid), VI & XII (cranial nerves), Tr (trapezoid body). A-E correspond to rostrocaudal levels shown in Figure 2.


VI
M
Tr
A
B
C
D
E
S
P
L
XII

et al., 1982], and derangements in CNS GABAergic function have been suggested in the pathogenesis of a variety of disease syndromes such as epilepsy [Meldrum, 1975; Iadarolla and Gale, 1983], Huntington's chorea [McGeer and McGeer, 1976; Enna et al., 1976], Parkinson's disease [Hornyekiewicz et al., 1976], schizophrenia [Van Kammen, 1977], tardive dyskinesia [Tell et al., 1981], and several cardiovascular disorders [DeFeudis, 1981].

Current evidence suggests that GABA is important in modulating the peripheral cardiovascular system. Takahashi and colleagues [1955] were the first to investigate the cardiovascular reponses produced by GABA. They injected GABA into an ear vein in rabbits and noted a dose-dependent fall in blood pressure. Peak effects occurred in 10-15 seconds after injection and persisted about ten minutes. Bradycardia was also observed. In a subsequent report [Takahashi et al., 1959], this group investigated the site of GABA action in rabbits, dogs, cats, and isolated toad hearts. A sequence of experiments indicated that GABA did not have a direct peripheral action, so they examined the possibility of a central action. Injection into the vertebral artery produced the shortest latency in blood pressure decreases as compared to injection into the external jugular vein, submaxillary artery, or internal carotid artery, suggesting that the site of action was directly supplied by the vertebral artery. Intracisternal injections also produced hypotension and bradycardia. The hypotension was prevented with ganglionic blockade and transection below the level of the obex, but not by transection rostral to the acoustic tubercle. They concluded that GABA produced its effects on blood pressure and heart rate in the medulla oblongata.

Over the next twenty years there were many studies done investigating the central role of GABA in cardiovascular regulation by various routes of injection of GABA, GABA agonists and antagonists, and in a variety of mammalian species (mice, rats, cats, rabbits, and dogs) [Elliott and Hobbiger, 1959; Stanton and Woodhouse, 1960; Stanton, 1963; Bharghava et al., 1964; Sgaragli and Pavan, 1972; Guertzenstein, 1973; Philippu et al., 1973; DiMicco et al., 1977a,b; Antonaccio and Taylor, 1977]. As a result, both forebrain and hindbrain mechanisms of the GABA-induced hypotension have been suggested.

The evidence for a major forebrain mechanism was presented in studies in which the GABA antagonists, picrotoxin or bicuculline [Olsen et al., 1979], were injected into cats. GABA blockade caused hypertension, increased hindlimb vascular resistance, tachycardia, and/or ventricular dysrhythmias when administered into and restricted to the lateral and third ventricles of cats [DiMicco et al., 1977b; DiMicco and Gillis, 1979; Williford et al., 1980a; DiMicco, 1982; Schmidt and DiMicco, 1984]. These effects resulted from disinhibition in the periventricular forebrain of both tonically active sympathoexcitatory pathways and of descending vagal inhibitory pathways. These forebrain systems appeared to be maximally activated by GABA since similarly administered muscimol, a potent GABA agonist [Johnston et al., 1968; Enna and Maggi, 1979], had no effect on cardiovascular variables but it prevented or reversed the effects of bicuculline [Williford et al., 1980a; DiMicco, 1982; Schmidt and DiMicco, 1984].

When GABA agonists were injected intracerebroventricularly and permitted access to the hindbrain the blood pressure and heart rate fell [Bhargava et al., 1964; Antonaccio and Taylor, 1977; Antonaccio

et al., 1978; Sweet et al., 1979; Persson, 1980b, 1983c; Bousquet et al., 1981b, 1982b, 1984; Antonaccio and Snyder, 1981; Baum and Becker, 1982; Brennan et al., 1983]. Furthermore, injections restricted to the fourth ventricle produced the same effects [Williford, et al., 1980b, Snyder and Antonaccio, 1980; Gillis et al., 1982a]. These effects of intracerebroventricular or fourth ventricle administration of GABA agonists were sensitive to reversal or blockade by picrotoxin or bicuculline [Antonaccio and Taylor, 1977; Antonaccio et al., 1978; Williford et al., 1980b; Persson, 1980b; Bousquet et al., 1981b, 1984; Antonaccio and Snyder, 1981]. These effects appeared to be the result of GABAergic inhibition of sympathetic outflow because decreases in blood pressure and/or heart rate were accompanied by decreases in sympathetic nerve discharge [Antonaccio and Taylor, 1977; Antonaccio et al., 1978; Snyder and Antonaccio, 1980; Antonaccio and Snyder, 1981; Baum and Becker, 1982], or were attenuated by prior systemic administration of reserpine [Persson, 1980b].

A site in the hindbrain for GABA's cardiovascular depressant effects therefore seemed likely. However, microinjections of GABA agonists into areas noted for their cardiovascular roles such as the A1 region [Blessing and Reis, 1982, 1983], the nucleus of the solitary tract (NTS) [Persson, 1981; Bousquet et al., 1982a] or the nucleus ambiguus [DiMicco et al., 1979; Blessing and Reis, 1983] caused hypertension. In contrast, hypotension and bradycardia were evoked by topical administration or by microinjection of GABAergic drugs into the VSMO of cats [Guertzenstein, 1973; Feldberg, 1976; Wennergren and Oberg, 1980; Bousquet et al., 1981a,b; Feldberg and Rocha e Silva, 1981; Yamada et al., 1982], suggesting that this was the region in

which GABA mediated its depressor effects in the hindbrain. Although there was no conclusive evidence, it was believed that GABA's effects were a result of inhibition of sympathetic outflow at the intermediate (S) area [Guertzenstein, 1973; Wennergren and Oberg, 1980; Feldberg and Rocha e Silva, 1981; Yamada et al., 1982]. The GABA system at the VSMO seemed to be distinct from forebrain systems. Muscimol topically applied to the VSMO caused hypotension in decerebrate and intact cats, and the hypotension was reversible with bicuculline [Yamada, 1982].

Further evidence that the VSMO was an important relay area in central sympathetic pathways came from stimulation and lesion experiments. Electrical stimulation of the ventral medulla increased blood pressure [Chai and Wang, 1962; Loeschcke et al., 1970; Trouth et al., 1973d; Neumayr et al., 1974; Guertzenstein and Silver, 1974; Dampney and Moon, 1980; Dampney, 1981; Dampney et al., 1982; Ross et al., 1983] and sympathetic nerve activity, and decreased femoral and renal vascular conductance [Dampney et al., 1982]. Since electrical stimulation excites cell bodies and also axons passing through an area, it was important to confirm that drugs known to influence the ventral medulla were acting on cell body/dendritic receptors. Later studies addressed this issue by administering excitotoxic amino acids such as glutamate or kainic acid. When injected into the CNS, these agents specifically excite cell bodies and dendrites as opposed to axons in passage or axon terminals, but are not selective for the type (neurotransmitter content, function) of cell bodies they excite. At higher doses, these agents are neurotoxic, but the mechanisms are poorly understood. It has been proposed that the neurotoxicity results from prolonged depolarization of neurons which eventually results in cell

death [Olney et al., 1971; Curtis et al., 1972; Buu et al., 1976; Schwarcz and Coyle, 1977; McGeer et al., 1978].

When glutamate or kainic acid were administered either topically or by microinjection into the ventral medulla (in the area which is also sensitive to GABA), hypertension resulted [Dampney, 1981; McAllen et al., 1982; Willette et al., 1983a; Ross et al., 1983, 1984a] suggesting that neuronal cell bodies mediated the action of drugs applied to this vasopressor area.

Conversely, non-specific electrical lesioning [Guertzenstein and Silver, 1974; Bousquet et al., 1975; Bloch et al., 1977; Dampney and Moon, 1980; Dampney, 1981; Granata et al., 1983a; Chalmers and West, 1983], cold block [Schlaefke and Loeschcke, 1967; Hanna et al., 1979; Schlaefke and See, 1980; Dembowsky et al., 1981], or application of tetrodotoxin [Bousquet et al., 1980; Ross et al., 1983] to this site decreased both blood pressure and electrical activity in thoracic white rami. Neurotoxic doses of kainic acid to the ventral medulla also resulted in very low blood pressures which persisted for hours [McAllen et al., 1982].

These functional studies of the VSMO (i.e. drug sensitivity, stimulation, and lesion experiments) indicated that the VSMO is a relay area involved with the tonic maintenance of sympathetic vasomotor and/or cardiac chronotropic tone.

The ventral medulla may also play a part in baroreflex mediation. The anatomic connections are compatible with such a role: There are direct projections from the NTS (where information from the baroreceptors and chemoreceptors in the carotid sinus and aortic arch makes its first synapse in the CNS) to the area near the VSMO [Loewy and

Burton, 1978; Errington and Dashwood, 1979; Andrezik et al., 1981b; Dampney et al., 1982], as well as reciprocal connections with the dorsal motor nucleus of the vagus (DMNX) [Errington and Dashwood, 1979] (site of vagal preganglionic neurons, and the cardiac vagus is soley responsible for the early phase of reflex induced bradycardia in the rat [Coleman, 1980]).

Physiologic studies lend further support the the hypothesis that in addition to maintenance of vasomotor tone, the VSMO may be the site of another synapse in the baroreflex arc. McAllen et al. [1982] studied the effects of lesions (kainic acid-induced) on the integrity of the baroreflex in a "blind sac" carotid sinus preparation in anesthetized cats. The major afferent pathways to the NTS were all severed except for one carotid sinus nerve which was stimulated by applying pressure to the carotid sinus (inflation with lactated Ringer's solution through the external carotid artery, with common and internal carotid arteries clamped). Stimulation (to 200 mmHg) caused typical decreases in systemic blood pressure, heart rate, and renal nerve activity of baroreflex activation. After chemically lesioning the S area with kainic acid bilaterally, these responses were abolished, and the authors concluded that cells near area S were involved in transmission of baroreceptor input to sympathetic vasomotor outflow. There was one major problem with this study: The lesions per se reduced blood pressure, heart rate and renal nerve activity to such extents, that further reduction by baroreceptor stimulation was probably not possible.

Granata et al. [1983a] tested this same hypothesis and devised a method for isolating the baroreceptor reflex arc while maintaining normal blood pressure levels in anesthetized rats. Their hypothesis was based

on the anatomical findings that projections from baroreceptors (and chemoreceptors) in the vagus nerve project to the NTS bilaterally [Kalia and Mesulam, 1980] and the NTS projects to the area of the VSMO unilaterally [Ruggiero et al., 1982]. Electrolytic lesions were placed in the NTS contalateral and VSMO area ipsilateral, to the side where baroreflex afferents were stimulated (either electrical stimulation of the central end of a cut vagus nerve or manual stretching of the carotid sinus area with a ligature around the common carotid artery). Lesion of the NTS caused an increase and lesion of the VSMO area caused a decrease in blood pressure which offset each other so that blood pressure and heart rate were not different from values before the lesions were placed. The reflex fall in blood pressure and heart rate were abolished after both lesions, implying that neurons in the NTS synapsing in (or projecting through, because the lesions also destroyed axons) the ventral medulla mediated the vasodepressor response from baro- and chemoreceptors.

Electrophysiological evidence supports the hypothesis that there is in fact a synapse of the baro- and chemoreflex arc in the ventral medulla [Ciriello and Caverson, 1984a,b; Caverson and Ciriello, 1984]. Stimulation of baroreflex afferents (carotid sinus nerve or aortic depressor nerve) orthodromically excited neurons (recorded from extracellularly) in the ventral medulla. Furthermore, these same neurons projected directly to the paraventricular and supraoptic nuclei in the hypothalamus (demonstrated by antidromic activation) suggesting that this ascending pathway may be involved in vasopressin release during activation of cardiovascular afferent fibers.

Functional neuroanatomical mapping of CNS areas that receive aortic baroreceptor afferent information was done by injecting [^{3}H]2-deoxyglucose intravenously while stimulating afferents for 45 minutes

(electrical stimulation of the aortic nerve of bolus intravenous injections of phenylephrine) in anesthetized rats [Ciriello et al., 1983]. The rats' brains were processed for autoradiographic localization of areas of increased density. Increased density (2-3 times control) was localized along the VSMO, extending 1 mm into the parenchyma, between the pyramidal and trigeminal tracts. The increased metabolic activity of this region implied from the study, supports a role of the VSMO in the baroreflex. (Note: comparable computerized densitometric values were noted in other appropriate areas also - ex: NTS, DMNX).

Finally, there is evidence for GABAergic control over the baroreflex at the VSMO. The responses to bilateral carotid artery occlusion (increased hindlimb muscle blood flow and renal blood flow) were reduced by 42% and 96% respectively after topical application of GABA to the S area of the VSMO in cats [Wennergren and Oberg, 1980]. GABA appears to be involved also in the vasodepressor component of the reflex. Application of GABA antagonists (bicuculline or picrotoxinin) to the S area of the VSMO produced a dose-dependent blockade of the baroreflex (especially the vasodepressor component) in the "blind sac" preparation described above [Yamada et al., 1984].

The ventral medulla may also be involved in other cardiovascular related reflexes. Production of cerebral ischemia in rabbits by total interruption of the blood supply to the brain (bilateral vertebral and common carotid artery occlusions), resulted in a pressor response of 95±17 mmHg that was reduced by 70% after bilateral electrolytic lesions in the region of the intermediate area [Dampney and Moon, 1980]. Production of the "defense reaction" in cats by electrical stimulation of areas in the hypothalamus and amygdala, resulted in increased blood

pressure, mesenteric vasoconstriction, skeletal muscle vasodilation, tachycardia, pupillary dilation, retraction of the nictitating membranes, and piloerection. Bilateral topical application of glycine to the intermediate area of the VSMO reduced all of these responses except the tachycardia. Furthermore, all of these responses could be elicited by electrical stimulation of the intermediate area [Guertzenstein et al., 1978].

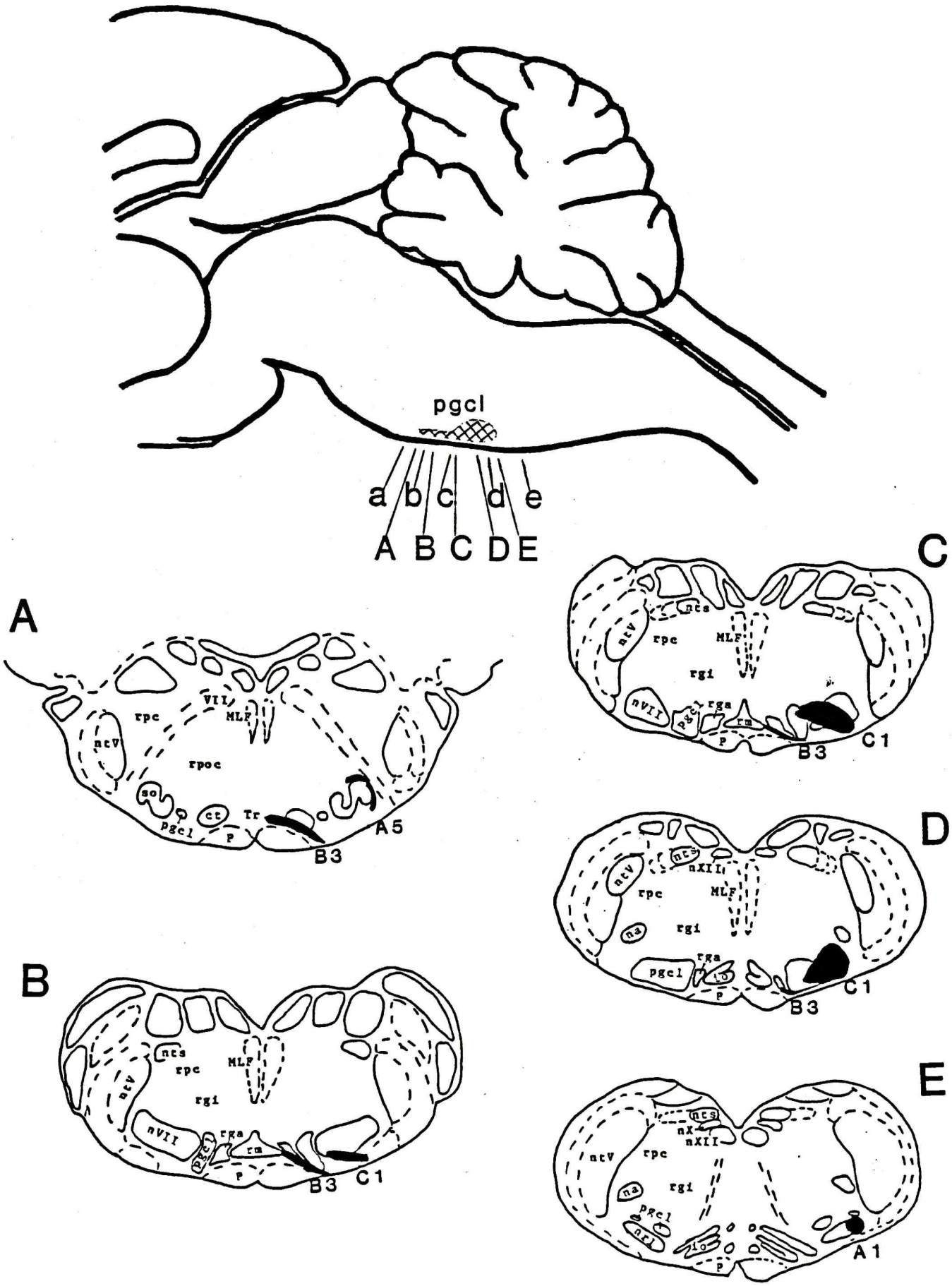
The results of these cardiovascular reflex related studies suggested that in addition to its role in tonic maintenance of vasomotor and chronotropic tone, the ventral medulla was also an important relay site for phasic contol of the cardiovascular system. Results from anatomic studies supported these hypotheses.

Morphologic studies showed the possible existence of neuronal substrates for the observed effects of drugs at the VSMO. Petrovicky [1968] described this area as having a very thin marginal glia with neurons directly under the pia mater. These observations were later extended by Liebstein et al., [1981] using a horseradish peroxidase (HRP) technique with light and electron microscopic visualization. HRP is taken up by axon terminals via a nonspecific pinocytotic mechanism, transported in vesicles to the soma where fusion with lysosomes occurs, and is enzymatically degraded to produce a visible reaction product. This group applied HRP to the VSMO in five rostrocaudal positions in cats, and then described two different cell types below the surface. Labeled cells in the S area contained fewer small and more large neurons than the M or L areas. The large cells showed maximal accumulation to a depth of 400-1000 μm below the surface, although small cells were labeled up to 80 μm from the surface. Most of the labeled neurons were in the lateral paragigantocellular nucleus (Figure 2). Ross and colleagues

Figure 2. Top: schematic diagram of a parasaggital section of a rat's brainstem with location of lateral paragigantocellular nucleus (PGCL). A-E: levels at which corresponding schematic coronal sections are shown below. a-e: levels at which corresponding coronal sections are shown in figure 17. A1,A5 = noradrenergic cell groups; B3 = lateral extensions of serotonergic cell group; C1 = adrenergic cell group; all shown in solid black on the right side of the coronal sections A-E.

ABBREVIATIONS

ct	NUCLEUS OF THE TRAPEZOID BODY
io	INFERIOR OLIVE
MLF	MEDIAL LONGITUDINAL FASCICULUS
na	NUCLEUS AMBIGUUS
nrl	LATERAL RETICULAR NUCLEUS
nts	NUCLEUS OF THE SOLITARY TRACT
ntV	NUCLEUS OF THE SPINAL TRIGEMINAL TRACT
nVII	FACIAL NUCLEUS
nX	DORSAL MOTOR NUCLEUS OF THE VAGUS
nXII	HYPOGLOSSAL NUCLEUS
P	PYRAMID
pgcl	LATERAL PARAGIGANTOCELLULAR NUCLEUS
rga	GIGANTOCELLULAR RETICULAR NUCLEUS, PARS ALPHA
rgi	GIGANTOCELLULAR RETICULAR NUCLEUS
rm	RAPHE MAGNUS
rpc	PARVOCELLULAR RETICULAR NUCLEUS
rpoc	CAUDAL PONTINE RETICULAR NUCLEUS
so	SUPERIOR OLIVE
Tr	TRAPEZOID BODY
VI	ABDUCENS NERVE
VII	FACIAL NERVE
XII	HYPOGLOSSAL NERVE


pgcl
a b c d e
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A1

[1981a] injected HRP into the spinal cords of rats and observed that the mean distance from the pial surface of retrogradely labeled cells at the VSMO was 12.9 μm. HRP reaction product to topically applied HRP and visualized with light and electron microscopy [Trouth et al., 1982] also revealed neurons close to the ventral surface (10 μm), as well as numerous synapses. Moreover, microvasculature was conspicuously absent from the marginal glia and diffusion of HRP into blood vessels was blocked at tight junctions, providing an anatomic basis for a blood-brain barrier (at least to HRP molecules - MW 40,000), and suggesting that drugs reaching this area exerted their effects locally.

The closest nuclear structure to the VSMO, and therefore the most likely area from which physiological responses are evoked is the lateral paragigantocellular nucleus (PGCL; Figure 2). A detailed account of its conformation and cytology in rats was described by Andrezik et al. [1981a]: The PGCL is cesta-shaped, with its caudal boundary at the junction of the rostral and middle thirds of the inferior olivary nucleus, and its rostral boundary at the level of the trapezoid body. Laterally it is bound by the lateral reticular nucleus (caudally) and facial nucleus (rostrally); medially it is bound by the pyramid; dorsally it extends about 1 mm from the VSMO. Most of the neurons are small (< 150 μm^2) and neurons of similar sizes tend to form subgroups between the rostral and caudal PGCL, with large neurons (> 250 μm^2) found mainly in the caudal portions.

The VSMO shares connections with other CNS regions that are thought to be involved in cardiovascular modulation, and these were determined by HRP retrograde and tritiated amino acid orthograde labeling, as well as lesion techniques. Afferent fibers projected from the

NTS [Loewy and Burton, 1978; Errington and Dashwood, 1979; Andrezik et al., 1981b; Dampney et al., 1982], DMNX [Errington and Dashwood, 1979], lateral hypothalamic area and paraventricular nucleus [Andrezik et al., 1981b], and parabrachial nucleus [Andrezik et al., 1981b]. Efferent fibers projected to the NTS and the DMNX in the medulla, and others ascended to the parabrachial nucleus and locus coeruleus in the pons, to the paraventricular nucleus, supraoptic nucleus, and dorsal and lateral areas of the hypothalamus, as well as the median eminence [Loewy et al., 1981; McKellar and Loewy, 1981].

Of particular importance are descending projections from the VSMO to the intermediolateral cell column (IML) of the spinal cord [Amendt et al., 1978, 1979; Martin et al, 1979; Loewy and McKellar, 1981; Loewy et al., 1981; Helke, et al., 1982; Caverson et al., 1983a; Miura et al., 1983; Ross et al., 1984b]. Because the IML is the site of origin of most sympathetic preganglionic neurons [Schramm et al., 1975; Rando et al., 1981; Gilbey et al., 1982; Holets and Elde, 1982; Haase et al., 1982; Luiten et al., 1984] (in the rat), these descending projections may have mediated the sympathetic responses elicited from the VSMO.

Recent electrophysiologic evidence supports the anatomic link between the VSMO and the IML. Basal renal sympathetic (post-ganglionic) nerve activity was temporally correlated with the firing times of ventrolateral medullary neurons, and electrical stimulation of the same neurons increased sympathetic nerve activity [Barman and Gebber, 1983]. A monosynaptic connection was suggested based on conduction velocities of antidromically activated neurons in the ventral medulla from the IML [Caverson et al., 1983].

Partial neurochemical characterization of the VSMO has been elucidated through immunohistochemical, specific neurotoxin, fluorescence, and lesion techniques. Many classes of neurotransmitters (ex: biogenic amines, amino acids, and peptides) are indigenous to the VSMO and are presented in Table 2.

With combinations of neurochemical and neuroanatomical techniques it is possible identify neuronal pathways and identify their neurotransmitters content simultaneously. Three such pathways were found to originate in the ventral medulla, terminate in the IML, and contain either serotonin [Loewy and McKellar, 1981], substance P [Helke et al., 1982], or epinephrine [Ross et al., 1984b]. Although such techniques have not been used yet to determine other transmitter specific pathways, the potential significance of elucidating the pathways from the brain to preganglionic sympathetic neurons is of utmost importance. While it is tempting to speculate that these serotonin, substance P, or epinephrine pathways may serve as neuronal substrates for the cardiovascular responses elicited by drugs acting at the ventral medulla, any of those neurotransmitters or putative neurotransmitters indigenous to the VSMO may serve this function. In addition to the potential candidates at the VSMO, transmitters have also been identified in the IML which may represent terminals of neurons originating in higher centers, possibly the VSMO, and are presented in Table 3. It is therefore possible that any of these neurotransmitters may be the specific neurotransmitter(s) that transmits the cardiovascular information from the VSMO to the IML of the spinal cord.

Thus, efforts have been made to ascribe the inferred sympathoexcitatory function to the pathway from the VSMO to the IML, with special

Table 2

NEUROCHEMISTRY OF THE VENTROLATERAL MEDULLA

acetylcholinesterase	Palkovits and Jacobowitz, 1974; Bowker et al., 1983; Satoh et al., 1983
norepinephrine	Dahlstrom and Fuxe, 1964, 1965; Fuxe, 1965; Palkovits and Jacobowitz, 1974; Satoh et al., 1977
epinephrine	Hokfelt et al., 1974; Howe et al., 1981a,b; Granata et al, 1983a; Ross et al., 1981b, 1983, 1984a
serotonin	Dahlstrom and Fuxe, 1964,1965; Hokfelt et al., 1978; Chan-Palay et al., 1978; Loewy and McKellar, 1981; Steinbusch, 1981; Johansson et al., 1981; Bowker et al., 1982a.b, 1983; Gilbert et al., 1982; Hunt and Lovick, 1982; Howe, et al., 1983a
GABA	Meeley et al., 1984
avian pancreatic polypeptide	Hunt et al., 1981
bovine pancreatic polypeptide	Olschowka et al., 1981
corticotropin releasing hormone	Olschowka et al., 1982
enkephalins	Hokfelt et al., 1979; Finley et al., 1981b; Hunt and Lovick, 1982; Khachaturian et al., 1983; Pickel et al., 1983
β-lipotropin	Hunt and Lovick, 1982
neuropeptide Y	Hokfelt et al., 1983b
neurotensin	Beitz, 1982
proctolin	Holets et al., 1984
somatostatin	Finley et al., 1981a
substance P	Hokfelt et al., 1978; Ljungdahl et al., 1978a; Chan-Palay et al., 1978; Johansson et al., 1981; Bowker et al., 1982a, 1983; Gilbert et al., 1982; Pickel et al., 1983
thyrotropin releasing hormone	Hokfelt et al., 1975; Johansson et al., 1981; Bowker et al., 1982, 1983; Gilbert et al., 1982

Table 3

NEUROCHEMISTRY OF THE IML

norepinephrine	Dahlstrom and Fuxe, 1965; Fuxe, 1965; Zivin et al., 1975; Ljungdahl et al., 1978b; Fleetwood-Walker and Coote, 1981; Westlund et al., 1983, 1984
epinephrine	Hokfelt et al., 1974; Zivin et al., 1975; Fleetwood-Walker and Coote, 1981; Sangdee and Franz, 1983; Caserta and Ross, 1983
dopamine	Zivin et al., 1975; Fleetwood-Walker and Coote, 1981
serotonin	Dahlstrom and Fuxe, 1965; Fuxe, 1965; Zivin et al., 1975; Loewy and McKellar 1981; Steinbusch, 1981; Gilbert et al., 1982; Holets and Elde, 1982, 1983; Kojima and Sano, 1983
angiotensin II	Fuxe et al., 1976
avian pancreatic polypeptide	Hunt et al., 1981
corticotropin releasing hormone	Merchenthaler et al., 1983
enkephalins	Holets and Elde, 1982,1983; Przewlocki et al., 1983; Romagnano and Hamill, 1984
oxytocin	Swanson and McKellar, 1979; Sofroniew, 1980; Holets and Elde, 1982, 1983
proctolin	Holets et al., 1984
somatostatin	Forssmann et al., 1979; Holets and Elde, 1982, 1983
substance P	Ljungdahl, 1978a,b; Johansson, 1981; Gilbert et al., 1982; Helke et al., 1982; Holets and Elde, 1982, 1983; Ho, 1983; Przewlocki, 1983
thyrotropin releasing hormone	Hokfelt et al., 1975a; Gilbert et al., 1982; Lechan et al., 1983
vasopressin	Sofroniew, 1980

emphasis on the role of the three identified neurotransmitter specific pathways (serotonin, substance P and epinephrine) and will be described. Logical approaches to this problem included stimulation or blockade of this pathway either in the ventral medulla or spinal cord, and monitoring either pre- or postganglionic nerve activity, peripheral catecholamine levels, blood pressure and/or heart rate. Pharmacologic manipulations of these approaches with appropriate administration of agonists, antagonists, and neurotoxins have helped to impute putative cardiovascular function to these neurotransmitters. Each case for a serotonin-, substance P-, or epinephrine-containing VSMO/PGCL-IML pathway mediating sympathoexcitatory cardiovascular effects is presented:

Serotonin

Serotonin (5-hydroxytryptamine; 5-HT), a vasoconstrictor substance isolated from beef serum, was chemically characterized in 1949 [Rapport, 1949] and identified in the brain in 1953 [Twarog and Page, 1953]. Dahlstrom and Fuxe [1964, 1965] were the first to report that cell bodies in the brainstem fluoresced for 5-HT and projected to the spinal cord. Based on the various aggregations of 5-HT neurons, they devised a nomenclature for these cell groups, B1-B9. The lateral extension of the B3 cell group is the area that most closely corresponds with the lateral paragigantocellular nuelei (PGCL) [Taber, 1961; Andrezik et al., 1981a], and underlies area S, or the intermediate area of the VSMO (Figure 2). Therefore, the demonstration of 5-HT at both the VSMO and the IML (see Tables 2 and 3) suggested that 5-HT might be a mediator of the sympathoexcitatory cardiovascular effects in the VSMO-IML pathway. Loewy and McKellar [1981] produced the first evidence of such a pathway per se: HRP was injected into the first two thoracic levels of rats'

spinal cords. After two days survival time, sections of the medullae were processed for retrograde HRP reaction product and 5-HT histofluorescence. Double-labeled (both HRP and 5-HT) cells were seen in the ventral medulla underlying areas S and L as well as the medial raphe nuclei. These results indicated that a 5-HT pathway projected from the ventral medulla to the spinal cord, however the injection site was not restricted to the IML. In the second series of experiments, ^{3}H-labeled amino acids were injected into the ventrolateral medulla (sites of double labeled cells) and resulted in heavy orthograde labeling bilaterally (with an ipsilateral predominance) in the IML of the thoracic and upper lumbar spinal cord. This labeling was absent in rats pretreated with intracerebroventricular injections of 5,6- or 5,7-dihydroxytryptamine, 5-HT neurotoxins, but still present in rats pretreated with intracerebroventricular injections of 6-hydroxydopamine, a catecholamine neurotoxin. Taken together, these two series of experiments showed evidence for a 5-HT pathway that could mediate the cardiovascular effects produced by VSMO stimulation.

The relationship between immunofluorescent 5-HT fibers and sympathetic preganglionic neuronal innervation of the adrenal gland has been studied in rats [Holets and Elde, 1982] and kittens [Holets and Elde, 1983]. Injection of the fluorescent retrograde tracer, Fast Blue, into rats' adrenal medullae produced labeling of cell bodies in the IML at all segments. Dense immunofluorescent labeling of 5-HT fibers were seen in the IML surrounding retrogradely labeled (and unlabeled) cell bodies, suggesting 5-HT innervation of sympathetic preganglionic neurons that projected to the adrenal gland. Cervical (7^{th} segment) transections of the spinal cord caused total depletion of 5-HT in the IML to

the 5th thoracic segment, but there was still some immunoreactivity below this segment. Cervical hemisections caused a decreased density of 5-HT labeling below the lesion, bilaterally. These results suggested that neurons which innervate the adrenal medulla receive ipsilateral and contralateral 5-HT input, partially from supraspinal and partially from intraspinal origin.

If 5-HT is a neurotransmitter of the VSMO-IML pathway, then one would expect excitatory responses to various methods of stimulating the system, and decreased cardiovascular activity following blocking drugs or serotonin neurotoxins. Indeed, iontophoretic application of 5-HT to antidromically identified SPN increased their firing rate [deGroat and Ryall, 1967; Coote et al., 1981; McCall, 1983]. These responses were blocked by prior iontophoretic or intravenous administration of the 5-HT antagonists, methysergide and metergoline. Furthermore, the 5-HT antagonists alone depressed the spontaneous discharge rate of sympathetic preganglionic neurons in intact but not in animals with cervical spinal transections. These data suggest a tonic excitatory input from supraspinal 5-HT neurons [McCall, 1983], consistent with the nature of the VSMO-IML pathway.

Howe et al. [1983b] provided evidence that 5-HT was the mediator of pressor effects evoked by VSMO stimulation. Electrical stimulation of the B3 cell group caused intensity and frequency dependent increases in blood pressure. In rats whose thoracic spinal cord 5-HT had been depleted to 10% of normal by pretreatment with intraspinal 5,7-dihydroxytryptamine, the evoked increases in blood presssure were reduced by more than 50%.

Thus, the cardiovascular role of 5-HT in the VSMO-IML pathway,

and whether it was under tonic GABA inhibition at the VSMO, was still undetermined.

Substance P

Substance P (SP) was first detected in extracts of horse intestines and brains and shown to have hypotensive and smooth muscle contracting properties [von Euler and Gaddum, 1931]. It was prepared in powder form, called preparation P, and later given the name "substance P" [Gaddum and Schild, 1934]. SP was subsequently purified from bovine hypothalamus and chemically characterized as an undecapeptide with the amino acid sequence Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 [Chang and Leeman, 1970; Chang et al., 1971]. The uneven distribution of SP in the CNS was originally reported by Kopera and Lazarini [1953] and detailed immunohistochemical mapping of its distribution in cell bodies and nerve terminals in rats has since been presented [Ljungdahl et al., 1978a,b; Cuello and Kanazawa, 1978].

The presence of SP cell bodies in the ventral medulla, terminals in the IML (see Tables 2 and 3), suggested the possibility of a SP-containing bulbospinal pathway which was confirmed by Helke and colleagues [1982]. This group studied the effect of various unilateral medullary electrolytic lesion on SP content (measured by radioimmunoassay) in rats' spinal cords. Lesions of the ventral medulla decreased SP content 38% in the IML. They concluded that a SP-containing bulbospinal pathway projected bilaterally to the IML, however the origin of this pathway in the ventral medulla appeared to be caudal to the VSMO/PGCL. Because of the large extent of the lesions (at least 2 mm transverse diameter) it is probable that the caudal lesions included a portion of the VSMO/PGCL.

Lesions in the midline raphe nuclei and the nucleus reticularis gigantocellularis, pars α(area of the lateral B3 cell group) did not change SP levels in the IML. Functional studies supported the existence of a SP-containing VSMO-IML sympathoexcitatory pathway involved with cardiovascular function. Iontophoretic application of SP in the IML increased the firing rate of antidromically identified SPN in rats [Gilbey et al., 1983] and cats [Backman and Henry, 1984], consistent with the excitatory nature of this pathway. In addition, intrathecal administration of SP in rats increased plasma levels of epinephrine and norepinephrine; effects which were prevented by similar administration of a SP antagonist [Yashpal et al., 1983]. That intrathecal administration of SP antagonist alone lowered the blood pressure to levels of a cervically transected rat, suggested that SP exerted tonic excitatory effects on the cardiovascular system [Loewy and Sawyer, 1982].

Additional pharmacologic findings indicated that the ventral medulla was the origin of these effects of SP in the spinal cord. Topical application of kainic acid to the combined intermediate and caudal area of the VSMO in rats increased the blood pressure and heart rate by about 80 mmHg and 50-140 beats/minute, respectively. These effects were reversed or blocked by intrathecal administration of a SP antagonist [Loewy and Sawyer, 1982]. Takano et al., [1984a] microinjected kainic acid into the ventral medulla (injection site included areas underlying the combined intermediate and caudal VSMO), while recording blood pressure and collecting spinal cord superfusate samples that were subsequently assayed for SP (by radioimmunoassay). They reported temporally related increases in blood pressure (greater than 200 mmHg) and increases in SP in the superfusates (100%).

The anatomic relationship between immunofluorescent SP fibers and sympathetic preganglionic neurons to the adrenal gland was also examined [Holets and Elde, 1982]. True Blue, a fluorescent retrograde tracer, was injected into the adrenal medulla of rats and caused labeling of cell bodies in the IML from the first thoracic to the second lumbar segments. Many of these cell bodies were surrounded by immunoreactive SP fibers, suggesting that SP neurons projecting to the IML specifically innervated SPN to the adrenal medulla. Furthermore, in rats whose spinal cords had been hemi- or transected at the 7^{th} cervical segment, there was a bilateral decrease in the density of SP-immunoreactive fibers in the IML caudal to the transection (absolute absence of SP immunororeactive fibers after total transections), suggesting that SP fibers in the IML originate in supraspinal areas.

SP receptors have been identified in the thoracic spinal cord of rats and their localization to sympathetic nuclei suggests that SP might be released from terminals in these regions. SP receptors were labeled with either ^{125}I-Bolton-Hunter-SP [Charlton and Helke, 1984b] or ^{3}H-SP [Maurin el al., 1984] and the autoradiographic distribution was visualized by light microscopy. High densities of binding sites were localized to the IML and to the region of the central canal (another area where sympathetic preganglionic neurons enamate [Petras and Cummings, 1972]). Helke et al. [1984b] provided more direct evidence that the SP receptors in the IML are located on sympathetic preganglionic neurons per se. Ricin was injected unilaterally into the superior cervical ganglion of the paravertebral sympathetic chain in rats (ricin is a toxic lectin that is transported retrogradely to neuronal soma and causes cell destruction by inactivating ribosomes [Wiley et al., 1982]). After two

weeks survival, the spinal cords were sectioned and examined for cell loss as well as autoradiographic localization of ^{125}I-Bolton-Hunter-SP labeled receptors. Ricin injections reduced both the number of cell bodies and SP binding in the IML, demonstrating that SP binding sites are in fact located on cell bodies of sympathetic preganglionic neurons.

With respect to cardiovascular function, the binding kinetics of SP in homogenates of spinal cord dissections that included the IML and SP immunoreactivity in the IML, were compared in 4 and 16 week old normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) [Takano et al., 1984b]. The IML of SHR and WKY (16 week old) showed a single saturable high affinity (K_d 1.21 - 1.25 nM) binding site, but a higher number of binding sites was reported for SHR (B_{max} 24.5 vs. 19.9 fmol/mg protein for WKY). Similarly, the IML of SHR contained 20% more SP immunoreactivity than WKY. There were no differences in 4 week old rats.

Thus, there was a good indication that SP might be a neurotransmitter in the VSMO-IML pathway. The discrete origin of this pathway and whether it was tonically inhibited by GABA at the VSMO were undetermined, and the cardiovascular role of IML-SP had not been studied.

Epinephrine

Epinephrine was "discovered" in adrenal gland extracts in 1895 [Oliver and Shafer, 1895], isolated and characterized in 1899 [Abel, 1899], and its presence in the CNS was first demonstrated by bioassay in 1946 [von Euler, 1946]. Hokfelt et al. [1974] provided the first morphologic evidence for epinephrine in the CNS, based on the immunohistochemical staining of pheylethanolamine-N-methytransferase (the enzyme that catalyzes the formation of epinephrine from norepinephrine; PNMT). They

found two distinct groups of adrenergic cells, only in the medulla, which they coined the C1 and C2 cell groups. The distribution of cells in the C1 group closely corresponds with the conformation of the PGCL, and again, underlies the intermediate area of the VSMO. Comparing the locations of the C1 and B3 (lateral extensions) cell groups, the B3 area is more medially situated than the C1 area, however the intermediate area superficially and the PGCL in the ventral medulla contain the greater portion of both cell groups. In the same study, a high density of PNMT positive nerve terminals were found in the IML. The evidence that the C1 area in fact projected to the thoracic spinal cord and IML was provided by a series of anterograde and retrograde transport studies [Ross et al., 1981b, 1983, 1984b; Goodchild et al., 1984]. Injections of True Blue, Fast Blue, or HRP into the thoracic spinal cord produced retrograde labeling within the PGCL and near the VSMO, and many of these same cells were also immunohistochemically PNMT-positive. Injections of tritiated amino acids into the PGCL area produced anterograde labeling that was restricted to the IML as well as the nuclei IML pars funicularis, intercalatus (other sites of sympathetic preganglionic neurons [Petras and Cummings, 1972]) and intermediomedialis. This labeling correlated well with the distribution of PNMT-labeled terminals in the thoracic cord. After combined injections of tritiated amino acids in the PGCL area and HRP in the adrenal medulla, silver grains could be seen overlying previously photographed HRP-labeled SPN in the IML. These latter experiments suggested the potential for IML innervation by neurons in the PGCL, although not neurotransmitter-specific.

The evidence for the adrenergic VSMO-IML pathway being sympathoexcitatory and involved in cardiovascular function was highly correlative.

Electrical or chemical (glutamate, kainic acid, or bicuculline) stimulation in the "PNMT-positive area" of the ventral medulla evoked increases in blood pressure, heart rate [Ross et al., 1983, 1984a,b; Goodchild et al., 1984], and plasma catecholamines and vasopressin [Ross et al., 1984a]. Similar microinjections of GABA or tetrodotoxin, or electrolytic lesions caused decreases in blood pressure and heart rate. Howe et al. [1981a] and Chalmers et al. [1984] provided the only evidence that epinephrine-synthesizing neurons in the ventral medulla per se, might be responsible for elevating blood pressure. This group compared PNMT variables in 4 week and 4 month old WKY, SHR, and stroke prone spontaneously hypertensive rats (SPR) and reported that: 1) Four week old SHR and SPR had 20-32% more immunohistochemically identified total (C1, C2, and a newly classified medially situated C3 [Howe et al., 1981b]) PNMT-positive cells in the medulla than WKY. There were no differences in 4 month old rats. Regionally, C1, C2, and C3 areas each showed similar increases in numbers of PNMT-positive cells. 2) PNMT enzyme activity (15-31 pmol/mg protein/hour) was elevated 29-50% in SHR and SPR compared to WKY at 4 weeks and 4 months of age in medullary and thoracic spinal cord, but not hypothalamic homogenates. Similar findings were reported by Lew et al. [1979] and Saavedra et al. [1978]. There were no differences in 4 month old rats. 3) PNMT enzyme protein content was elevated in the medulla of 4 week old SHR and SPR compared to WKY. 4) The elevated blood pressure in 4 month old SHR and SPR compared to WKY correlated with the elevated PNMT activity in the ventral medulla and spinal cord. These correlations provided evidence that epinephrine in bulbospinal regions may be involved in the elevation of blood pressure.

However, other groups showed more directly that spinal cord epinephrine was inhibitory to the sympathetic nervous system. Microiontophoretic application of epinephrine to sympathetic preganglionic neurons decreased their firing rate or had no effect [Guyenet and Cabot, 1981; Coote et al., 1981; Guyenet and Stornetta, 1982]. Further support for an inhibitory role of epinephrine was reported by Sangdee and Franz [1983] who used selective-PNMT inhibitors to characterize the effects of endogenous epinephrine in spinal pathways projecting to the IML. They recorded evoked discharges from thoracic preganglionic rami to stimulation of the dorsolateral funiculus (the major route of descending axons to the IML), in cats with high cervical cord transections. Intravenous injection of PNMT inhibitors produced a gradual (maximum effect in 4 hours) and marked increase in the size of evoked responses (200-250% of control). There were two problems with this study: 1) The time course of measured inhibition of central epinephrine synthesis [Goldstein et al., 1980; Fuller et al., 1981] is about 1 hour. 2) These investigators did not verify decreased epinephrine levels in the spinal cord; and in fact the reported increases in the responses could have been a result of some other descending non-epinephrine pathway, which, over time would be depleted of its neurotransmitter by the transection.

The functional role of the adrenergic C1-IML pathway therefore remains undefined. Neuropeptide Y was shown to coexist with C1 cells [Hokfelt et al., 1983b] which, if released from terminals in the IML, may be the pressor substance of C1 neurons. Until more functional studies are done to evaluate the role of the C1-IML pathway in cardiovascular function, it is still a contender for a mediator of the sympathoexcitatory responses evoked by VSMO/PGCL stimulation under GABAergic inhibition.

Specific Aims

My intial goal was to determine if it was possible to study the role of the VSMO in cardiovascular regulation in a rat model. Although most of the previous studies involving the VSMO were done in larger animals, the potential advantages of this small animal model were numerous: Functional data from a rat can be more appropriately integrated with already available neuroanatomical and neurochemical data which was obtained largely from the rat. Characterization of a neuronal pathway necessitates knowledge of three elements (functions, anatomy, and transmitter content), and only after these elements are elucidated can the pathway be properly manipulated to investigate the role it might play in disease states or actions of various drugs. In addition, rats are relatively inexpensive research animals which allows many preliminary experiments to be done. Because rats are inbred for research purposes, they show less interanimal variability than mongrel cats and dogs. Rats are more resistant to infection than larger animals which provides a distinct advantage for chronic experiments. And finally, there are several genetically or drug-induced diseased rat models available (ex: hypertensive, stroke-prone, obese, diabetic, vasopressin deficient) if one wished to compare these to normal rats.

The first objective was to develop the expertise to perform the delicate surgical exposure of the VSMO and monitoring procedures in this small animal model.

The second objective was twofold: 1) To find out if the rat responded to pharmacological manipulations of the VSMO similar to the more frequently studied larger animals; and 2) to pharmacologically characterize in detail, a system which would be useful in future studies. Because

of its well characterized effects on cardiovascular endpoints in other species, a GABAergic system at the VSMO was investigated.

My second major goal was to determine, pharmacologically, which major neurotransmitter(s) pathway emanating from the VSMO was responsible for imparting information to the cardiovascular system. It was determined from the GABA studies that this information had to traverse the IML, so efforts were concentrated in the spinal cord. The evidence for 5-HT and SP as candidate transmitters in bulbospinal sympathetic pathways prompted an investigation into their possible link with the GABAergic drug-induced cardiovascular effects at the VSMO.

Within this second goal, the first objective was to determine which neurotransmitter antagonists/neurotoxins, administered intrathecally, could block the cardiovascular effects caused by GABAergic disinhibition at the VSMO.

Based on the results of the first objective, the second objective was to pharmacologically verify these results by characterizing the responses to intrathecally administered agonists, as well as evaluating agonist-antagonist interactions.

The third objective was to verify that the cardiovascular responses elicited by agonist activation in the spinal cord were in fact mediated by the sympathetic nervous system.

In summary, these studies were designed to characterize more fully the pathway that links the sensitive VSMO with the neurons directly involved in the control of vasomotor tone and heart action. Indeed, the results of these studies have theoretically important implications: 1) They may give insight to the etiologies of a variety of cardiovascular pathologies. 2) They may provide a better understanding of mechanisms

of action of drugs used today in treating cardiovascular diseases. 3) They may provide a better understanding of the mechanisms of cardiovascular side effects produced by drugs used to treat diseases unrelated to the cardiovascular system. 4) They may lead to the development of more specific drugs in the treatment of cardiovascular related pathologies.

METHODS

In vivo preparation and monitoring procedures

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY or Hilltop Lab Animals, Scottdale, PA) weighing 225-450 g were used in all studies. Tap water and standard rat chow (R/M/H 3000, Agway, Inc., Ithica, NY) were provided ad libitum, and rats were housed 4/cage at constant temperature on a 12 hour light/dark schedule. Rats were anesthetized with chloralose and urethane (60 and 800 mg/kg, respectively, i.p., dissolved in phosphate buffered saline (PBS; pH 7.4), in a volume of 3 ml/kg), unless otherwise stated. The rats were artificially ventilated on room air with a Harvard 680 rodent ventilator via a U-shaped tracheal cannula (PE-240). Ventilation was adjusted to maintain arterial pH within normal limits, by delivering a tidal volume of 9-14 x body weight (kg) and a rate of 60-64 breaths/minute. A femoral artery was cannulated (22-ga, Jelco angiocath) for measurement of blood pressure (Statham transducer) and withdrawal of blood samples. Arterial pH and gases were determined from 0.2 ml heparanized (1:1000 to coat the syringe barrel) samples with a Radiometer microsystem (BM 53 MK2) or Radiometer (ABL 3). Mean arterial determinations were pH 7.404 ± 0.002, pCO_2 34.7 ± 0.9, pO_2 90.7 ± 0.7. A femoral vein was cannulated (PE-50) for administration of drugs and fluid replacement. Intravenously administered drugs were dissolved in PBS and delivered in a volume of 1 ml/kg. Colonic temperature was maintained at 37 ± 1.5°C (Yellow Springs Instruments 402 probe and heating pad). Blood pressure, ECG (lead II), heart rate (cardiotachometer triggered from ECG), and temperature were all recorded on a Beckman R511A physiograph which was calibrated daily. PBS preparation is provided in the "Solutions" section.

Surgical exposure of the ventral surface of the medulla and drug administration

A clear surgical field was initially insured by using a U-shaped tracheal cannula for the tracheostomy so that the tubing from the ventilator was connected to the cannula via a Y-piece at chest level. The rat was placed in a David Kopf stereotaxic apparatus with the nose-piece at -10mm to stabilize the head in a supine position, and the procedure was facilitated with a Bausch and Lomb stereomicroscope (magnification range 4-20 X) and fiberoptic light source (American Optical). The sternohyoideus muscles were cut at their insertions and reflected caudally. The rostral part of the trachea and corresponding portion of the esophagus were ligated, cut at one tracheal ring rostral to the tracheostomy, and retracted rostrally. The longus capitis muscles were freed from their insertions in the basal occipital bone, and retracted caudally. The basal occipital bone was removed by drilling a "window" along its exposed borders with a Dremel motoflex drill and bit (105), then the dura and arachnoid were incised and reflected laterally. This procedure resulted in exposure of the ventral medulla from the pontomedullary junction to the caudal extent of the hypoglossal rootlets, and up to 3 mm lateral to the midline.

Drugs were dissolved in artificial cerebral spinal fluid (CSF) [Merlis 1940; Feldberg et al., 1970]. In the initial GABA sensitivity experiments, GABA (0.234 µmol) was injected in a volume of 10 µl over the entire exposed ventral surface, and the lateral, rostral and caudal boundaries were packed with Teflon® cottonoids to limit spread of the drug. In all succeeding experiments, drugs were topically applied with filter paper pledgets (Whatman 1) and doses of drugs were calculated

according to dry:saturated (CSF) weight ratio of the pledgets. Dry: saturated weight = 0.29 mg based on several sizes of filter paper and several weight determinations. Example: a 1 x 1.5 mm pledget weighed 0.146 mg and 0.5 mg, dry and wet respectively, so each pledget held 0.354 µl. Because pledgets were applied bilaterally, doses refer to the amount of drug contained in two saturated pledgets. Pledget dimensions were 0.5 x 0.5 mm in the GABA and BMI localization experiments, 1 x 1.5 mm in all other experiments, and were applied immediately lateral (apposing) to the pyramids. Filter paper wicks (about 3 cm x 3 mm) were placed at the margins of the exposed VSMO to prevent CSF accumulation and drug dilution. The pH of all topically applied drugs was 7.3-7.5.

For drug administration, pledgets were left in place for 2 minutes on the VSMO then the VSMO was washed with CSF 10 µl. Preparation of the artificial CSF is provided in the "Solutions" section.

Intrathecal (i.t.) catheterization and drug administration

Intrathecal catheterization was a modified procedure of Yaksh and Rudy [1976]. Rats were placed in a David Kopf stereotaxic head holder elevated so that the cervical and thoracic spines were in a near vertical position. The skin was incised in the midline from ear level, caudally 2 cm. The trapezius, semispinales, and rectus capitis muscles were either cut bilaterally from their insertions in the occipital crest and retracted caudally, or retracted laterally. A 2 mm midline incision was made through the exposed atlanto-occipital membrane, the dura, and arachnoid. A loose knot was tied and cemented in a 9.0-9.5 cm PE-10 catheter that divided the catheter into 7 cm and 2 cm segments. The 7 cm end was inserted through the incision in the membrane into the spinal subarachnoid space along the spinal cord, until the knot stopped at the

membrane. The 2 cm end was brought out through the skin and the skin closed. At the end of each experiment, the dorsal vertebral column was removed and this consistently showed the catheter tip to be at the T_{10-11} vertebral levels.

Drugs were dissolved in a PBS vehicle and administered over 0.5 -3 minutes in a volume of 3.75-7.5 µl, plus 5.0-7.5 µl PBS to flush the catheter (total injection volume was 15 µl). The dead space volume of each catheter was about 5.0 µl. A hand-driven microdrive (David Kopf 1209) was used to deliver the 15 µl volume to the i.t. catheter, from a 1 ml syringe (Hamilton 1001TEFLL), via PE-90 connecting tubing that served as drug or vehicle reservoir. A small air bubble was drawn into the PE-90 tubing before the drug or vehicle, to note the progression of fluid delivery. The volume of fluid contained in the PE-90 tubing was about 5.0 µl/cm, so a total of 3 cm of fluid was injected. At the end of each experiment, 15 µl of a saturated solution of Fast Green or methylene blue dye was injected. Staining was seen from mid-cervical level to the caudal end of the spinal cord.

Decerebration technique

Each rat was given dexamethasone 0.4 mg (0.2 ml i.p.) to prevent brain edema, then placed in a David Kopf stereotaxic head holder with the nose-piece at -10 mm. The skull was opened along a transverse line immediately caudal to the lambda suture with a Dremel motoflex drill, and the dura was cauterized with a Valley Lab Surgistat. This completed the procedure for sham operated rats. For the decerebration the 2 cm x 2.5 x 0.3 mm cautery tip was directed about 17° rostrally and the midbrain transected by slowly advancing the tip until it hit the basal occipital bone. The tip was rotated medially and laterally, with care not to harm

the basilar artery. Drains (about 1.5 mm x 1 cm cut from a rubber glove) were inserted into the wound bilaterally, then a PBS-soaked gauze (about 1 cm x 0.5 cm) was placed over the wound, and the skin closed. Completeness of the mid- or inferior-collicular decerebration was verified by gross visual examination, after decapitation and removal of the brain at the end of the experiment.

[^{3}H]GABA distribution experiments

1. In vivo procedure.

Two pledgets, saturated with a solution of [^{3}H]GABA and unlabeled GABA in CSF, contained 0.2 µCi of [^{3}H]GABA and 0.78 µmol GABA. The pledgets were applied bilaterally to the intermediate area. At the peak of the cardiovascular responses (2 minutes), a peripheral blood sample (200 µl) was collected, the rat was immediately decapitated, the brain rapidly removed and frozen in powdered dry ice.

2. Microdissections

Coronal sections (300 µm) of the medulla (with cerebellum) were cut in a Minotone cryostat (-10°C; International Equipment Co.), then the frozen sections were dissected into 12 parts on the freezing stage of a Flexi-cool (FTS Systems, Inc.).

3. Quantitation

The dissected tissues were solubilized with Protosol (750 µl) in plastic scintillation vials (20 ml), covered with tin foil and incubated for 16 hours at room temperature. Ready Solv EP (10 ml) and glacial acetic acid (35 µl) were added to each vial, then the vials were loosely capped and placed in a Precision shaking water bath (37°C) for 80 minutes. Plasma (50 µl) was mixed with Ready Solv EP (10 ml) (preliminary experiments showed no differences in the amount of tritium between plasma

prepared as mentioned or whole blood that was processed according to New England Nuclear Instructions). The vials were capped and stored at 4°C for at least 24 hours before counting in a Beckman LS 7800 liquid scintillation counter.

4. Autoradiography.

Coronal sections (20 µm) of the medulla were cut in a Minotone cryostat (-12°C; International Equipment Co.), thaw mounted and vacuum dessicated. LKB Ultrofilm ^{3}H was exposed to the samples in an X-ray cassette for 4 or 8 weeks at room temperature. Adjacent tissue sections were stained with 0.1% thionin for histological verification.

5,7-dihydroxytryptamine (5,7-DHT) experiments and serotonin (5-HT) spectrofluorometric assay

Rats were anesthetized with halothane (2-4%) with oxygen (96-98%), 45-60 minutes after pretreatment with desmethylimipramine HCl to prevent 5,7-DHT uptake into catecholamine nerve terminals (25 mg/kg, dissolved in distilled water and injected in a volume of 1 ml/kg i.p.), and i.t. catheters were inserted. Rats received either 5,7-DHT 200 µg (free base) dissolved in 7.5 µl of 0.05% ascorbic acid in PBS (pH 4), or vehicle (adjusted to pH 4 with 1.0 N HCl), followed by 7.5 µl PBS flush. This drug regimen was repeated the next day, then the catheters were removed. *In vivo* experiments were done 10-14 days later, and at the end of each experiment, the animals were decapitated, their spinal cords removed and immediately frozen on powdered dry ice. The spinal cords were stored at -70°C until they were assayed for 5-HT. The 5-HT assay was a modified procedure of Hyyppä *et al.* [1973]. Thoracic spinal cord samples (70-108 mg) were homogenized in 2 ml acid butanol (0.85 ml concentrated HCl/1 butanol) and centrifuged (35,000 x g_{max} for 20 minutes). The

supernatants (1.7 ml each) were transferred to tubes containing 1.5 ml 0.1 N HCl and 6 ml heptane. The aqueous and organic phases were mixed by shaking for 10 minutes, then separated by centrifugation (1500 x g_{max} for 5 minutes). The organic phase was aspirated and discarded, and the aqueous phase containing 5-HT (1 ml) was transferred to tubes containing 100 µl 1% cysteine. O-phthaldehyde solution (6 mg dissolved in 150 ml concentrated HCl) 2 ml was added to each tube, then the tubes were heated at 100°C in a water bath, cooled to room temperature, and fluorescence was read at 355/470 (excitation/emission) nm in an Aminco-Bowman spectrophotofluorometer. Recovery for serotonin standard was 96%. Serotonin concentrations were expressed as ng/g frozen tissue weight.

Capsaicin (8-methyl-N-vanillyl-6-noneamide) experiments and substance P (SP) radioimmunoassay

On their second day of life, rats of both sexes received capsaicin 50 mg/kg subcutaneously in 0.1 ml, or the vehicle combination of ethanol, Tween 80, and PBS in a ratio of 1:1:8 in 0.1 ml [Jansco et al., 1977]. Two months later the in vivo experiments were done. At the end of each experiment, the thoracic spinal cord was rapidly removed, frozen on powdered dry ice, then stored at -70°C.

The micropunch technique and SP radioimmunoassay were procedures described by Helke et al. [1980, 1982] and Gillis et al. [1980]. Coronal sections (300 µm) of thoracic spinal cords were cut in a cryostat (-12°C), and the dorsal horns (DH), intermediolateral cell columns (IML), and ventral horns (VH) were dissected by the micropunch method of Palkovits [1973]. Samples consisted of four DH 500 µm, twelve IML 300 µm, or six-eight VH 500 µm diameter punches which were pooled from each rat, and placed into 0.4 ml plastic microtubes containing 100 µl

2 N acetic acid in an ice water bath. Samples were boiled for 10 minutes, returned to an ice water bath, sonicated (Kontes micro-sonic cell disrupter), and a 5-10 µl aliquot was removed for protein determination. Samples were centrifuged at 8900 x g_{max} Beckman microfuge B) for 3 minutes at 4°C, and duplicate 25 µl aliquots of the supernatant were removed and vacuum dried (Savant Speed Vac Concentrator) in 12 x 17 mm borosilicate glass tubes.

The radioimmunoassay: In an ice water bath, SP antibody (SP3B3 100 µl; final dilution of 1:40,000) and about 4000 cpm of [^{125}I]Tyr^{8}-SP (100 µl) were added to the dried samples and synthetic SP standards (5, 10, 20, 40, 80, 120, 200 pg each in 200 µl assay buffer in triplicate). Also included in the assay were triplicate tubes "total counts" and "non-specific binding" containing only [^{125}I]Tyr^{8}-SP (100 µl) and assay buffer (300 µl), and "total binding" containing [^{125}I]Tyr^{8}-SP (100 µl), SP antibody (100 µl), and assay buffer (200 µl). Assay buffer (200 µl) was added to all samples to bring the volume to 400 µl. The tubes were vortexed and refrigerated (4°C) for 24 hours. Normal rabbit serum (20% in PBS, pH 7.4; 25 µl) and sheep antiserum against rabbit γ-globulin (20 µl in 180 µl PBS) were added to all tubes except "total counts", the tubes were vortexed and refrigerated (4°C) for 24 hours. Samples were then centrifuged (1500 x g_{max} at 4°C for 15 minutes), the superatants poured off, and radioactivity of the pellets counted in a Micromedic Systems 4/600 gamma counter.

The rabbit antibody to SP (SP3B3) recognizes the C-terminal decapeptide-amide of SP. It shows 90% cross-reactivity with SP sulfoxide and negligible cross-reactivity with bombesin, eledoisin, physalaemin, met-enkephalin, and [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}]-SP.

Radioimmunoassay combined with HPLC [Helke et al., 1980; O'Donohue et al., 1981] resolved the immunoreactivity of this antibody for peptides in rat CNS into two components which coeluted with authentic SP and SP sulfoxide. Iodinated SP was prepared by the method of Mroz and Leeman [1979]. Assay sensitivity (10% displacement of the tracer) was 7 pg/tube. Protein content was determined by a modified method of Lowry et al. [1951]. Assay buffer preparation is provided in the "Solutions" section.

[pGlu^{5}, MePhe^{8}, MeGly^{9}]-SP(5-11) (DiME-SP) experiments and catecholamine radioenzymatic assay

At the peak of the DiME-induced (i.t.) pressor (7 minutes after injection), and tachycardic (10 minutes later) responses, blood samples (0.6 ml) were drawn and simultaneously replaced with heparinized donor blood. Each sample was immediately centrifuged (8800 x g_{max} for 5 minutes at 4°C in a Beckman Microfuge B), the plasma aspirated into 1.5 ml plastic tubes and stored at -70°C until assayed.

The catecholamine assay was done according to Durrett and Ziegler [1980]. Plasma samples were thawed and centrifuged (8800 x g_{max}; Eppendorf 5413) for 30 seconds, and kept in an ice water bath until the reaction incubation. Aliquots (100 µl) were transferred to 14 ml polypropylene (Sarstedt 538) tubes in duplicate. Also included in the assay were two "blank" plasma samples (no enzyme reaction mix), two "control" plasma samples, and catecholamine (norepinephrine, epinephrine, and dopamine) standards (125, 250, 500, 1000 pg each) in 0.01 N HCl, all in duplicate. Catecholamine standards (10 µl) were added to the appropriate tubes and extra set of blanks, controls, 250 and 500 pg standards were included after the samples. Ten µl of 0.01 N HCl were added to

the remaining tubes to bring the total volume to 110 µl. Catechol-O-methyltransferase reaction mix (100 µl) was added to all tubes except the blanks, then the tubes were incubated at 37°C for 90 minutes in a Precision shaking water bath. Tubes were returned to an ice water bath, borate buffer (200 µl; pH 10) and "cold carrier" (50 µl) were added and the tubes were vortexed. Tetraphenyl borate 1% (50 µl) was added and the tubes were vortexed. Toluene-isoamyl alcohol (7 ml) was added, the tubes were shaken for 5 minutes at room temperature, and centrifuged (1650 x g_{max}; IEC Centra-7R) at 18°C for 5 minutes. Tubes were placed in a dry ice/ETOH bath (4 at a time) until the aqueous phase was frozen solid, the organic phase was decanted into new 14 ml polypropylene (Sarstedt 538) tubes, each containing 0.1 N acetic acid (250 µl). The tubes were shaken for 5 minutes and centrifuged (1650 x g_{max}; IEC Centra-7R) at 18°C for 5 minutes. Toluene-isoamyl alcohol (3 ml) was added, the tubes were shaken, and centrifuged as above. Tubes were placed in a dry ice/ETOH bath (12 at a time), the organic phase was aspirated, and the aqueous layer was lyophylized overnight (VirTis 10-MR-TR lyophylizer; cold trap 10-100V at -60°C; vacuum 55 µmmHg at 25°C). "Cold carrier" (50 µl) was added to each lyophylate, the tubes were centrifuged (1650 x g_{max}; IEC Centra-7R) at 18°C for 30 seconds, then the samples were spotted onto preadsorbent thin layer chromatography (TLC) plates (7010-Si250F·PA (19C) J.T. Baker Chemical Co., Phillipsburg, NJ). Additional "cold carrier" (50 µl) was added to each tube and the TLC plates were respotted until all the sample was used up. The TLC plates were developed in TLC jars containing ethylamine solvint (105 ml) for 75 minutes. Sample bands were visualized, differentiated, and delineated with a pencil under ultraviolet light. The bands

corresponding to norepinephrine and epinephrine metabolites were scraped into separate 7 ml borosilicate glass minivials (Kimble). NH_4OH (2 N; 1 ml) was added to each vial and the vials were agitated by hand for 5 seconds. $NaIO_4$ (4%; 50 µl) was added, followed exactly 5 minutes later by glycerol (10%; 50 µl), and agitated by hand for 5 seconds. Acetic acid (10 N; 200 µl) and "Phosphor Only" (5 ml) were added, the vials were capped and shaken by hand vigorously, placed in a LKB Rack-Beta liquid scintillation counter for 2 hours, then counted for 5 minutes/vial. See "Solutions" section for details of the preparations.

Modified Lowry procedure for protein determination (micro technique)

At room temperature, sample aliquots (5-10 µl) were pipetted into 6 x 50 mm glass tubes (Kimax 45048), then NaOH 1 N (20 µl) was added to each tube (standards in quintuplicate and samples in duplicate) and vortexed. "Reagent C" (250 µl) was added to each tube, then 10 minutes later "Reagent E" (25 µl) was added and each tube was immediately vortexed. After 30 minutes, standards and samples were transferred to spectrophotometer microcells and absorbance read at 750 nm in a Gilford spectrophotometer. Standards (0, 0.625, 1.25, 2.5, 5.0, 7.5, 10 µg) were prepared from a stock bovine serum albumin solution (100 mg/100ml dH_2O). Assay sensitivity was 1.25 µg. Reagents "C" and "E" preparations are provided in the "Solutions" section.

Chemicals

γ-aminobutyric acid (Sigma Chemical Co., St. Louis, MO)

[^{3}H]γ-aminobutyric acid (s.a. 74 Ci/mmol, 692 mCi/mg; Amersham Arlington Heights, IL)

[^{3}H]S-adenosyl methionine (s.a. 12.2 Ci/mmol, 0.5 mCi/ml; New England Nuclear, Boston, MA)

atropine methylnitrate (Sigma Chemical Co., St. Louis, MO)

O-benzylhydroxylamine hydrochloride (Sigma Chemical Co., St. Louis, MO)

bicuculline methiodide (Pierce Chemicals, Rockford, IL)

bombesin (Peninsula Labs, Belmont, CA)

bovine serum albumin (Sigma Chemical Co., St. Louis, MO)

catechol-O-methyltransferase (spec. act. 4 μmol/mg N/10 minutes, purified from rat liver by the method of Nikodejevic et al., 1970)

capsaicin (Sigma Chemical Co., St. Louis, MO)

chloroform (Mallinckrodt, Inc., Paris, KY)

α-chloralose (Division Pharmacie, Clichy, France)

cinanserin (Squibb and Sons, Inc., Princeton, NJ)

L-cysteine (Sigma Chemical Co., St. Louis, MO)

desmethylimipramine (gift from Revlon Health Care Group, Tuckahoe, NY)

dexamethasone (Schering Veterinary, Kenilworth, NJ)

disodium ethylenediamine tetraacetate (EDTA; Fisher Scientific, Fairlawn, NJ)

5,7-dihydroxytryptamine creatinine sulfate (Sigma Chemical Co., St. Louis, MO)

L-epinephrine bitartrate (Calbiochem-Behring Corp., La Jolla, CA)

ethylamine (Sigma Chemical Co., St. Louis, MO)

ethyleneglycol-bis-(β-aminoethylether)N,N'tetraacetic acid (EGTA; Sigma Chemical Co., St. Louis, MO)

glutathione (reduced; Sigma Chemical Co., St. Louis, MO)

glycerol (Sigma Chemical Co., St. Louis, MO)

glycine (Sigma Chemical Co., St. Louis, MO)

halothane (Halocarbon Laboratories, Inc., Hackensack, NJ)

heparin sodium (porcine; Lypho-Med, Inc., Chicago, IL)

hydralazine (CIBA Pharmaceuticals, Summit, NJ)

3-hydroxytyramine hydrochloride (dopamine; Calbiochem-Behring Corp., La Jolla, CA)

isoamyl alcohol (Sigma Chemical Co., St. Louis, MO)

DL-metanephrine hydrochloride (Sigma Chemical Co., St. Louis, MO)

3-methoxytyramine hydrochloride (Sigma Chemical Co., St. Louis, MO)

L-norepinephrine bitartrate·2.5H_2O (Calbiochem-Behring Corp., La Jolla, CA)

normetanephrine hydrochloride (Sigma Chemical Co., St. Louis, MO)

muscimol (Sigma Chemical Co., St. Louis, MO)

pentolinium tartrate (Sigma Chemical Co., St. Louis, MO)

phentolamine (CIBA Pharmaceuticals, Summit, NJ)

phenylephrine hydrochloride (Sigma Chemical Co., St. Louis, MO)

O-phthaldehyde (Calbiochem-Behring Corp., Somerville, NJ)

PPO-POPOP (Liquifluor; New England Nuclear, Boston, MA)

procaine hydrochloride (Pfaltz and Bauer, Inc., Stamford, CT)

propranolol (Sigma Chemical Co., St. Louis, MO)

Protosol (New England Nuclear, Boston, MA)

Ready Solv EP (Beckman Instruments, Inc., Fullerton, CA)

sheep anti-rabbit serum (raised at NIH, Poolesville, MD)

sodium m-periodate (Sigma Chemical Co., St. Louis, MO)

strychnine sulfate (Sigma Chemical Co., St. Louis, MO)

substance P (Peninsula Labs, Belmont, CA)

[^{125}I]Tyr^{8}-substance P (prepared by the method of Mroz and Leeman, 1979)

[D-Pro^{2},D-Trp^{7,9}]-substance P (Peninsula Labs, Belmont, CA)

[D-Pro^{2},D-Phe^{7},D-Trp^{9}]-substance P (Peninsula Labs, Belmont, CA)

[D-Arg^{1},D-Pro^{2},D-Trp^{7,9},Leu^{11}]-substance P (Peninsula Labs, Belmont, CA)

[D-Pro^{4},D-Trp^{7,9,10}]-substance P(4-11) (Peninsula Labs, Belmont, CA)

[pGlu^{5},MePhe^{8},MeGly^{9}]-substance P(5-11) (Peninsula Labs, Belmont, CA)

substance P monoclonal antibody (Pel-Freez)

substance P polyclonal antibody (SP3B3; raised by us)

tetraphenyl boron (Sigma Chemical Co., St. Louis, MO)

toluene (Fisher Scientific, Fairlawn, NJ)

Tris(hydroxymethyl)aminomethane hydrochloride (Sigma Chemical Co., St. Louis, MO)

Tween 80 (Fisher Scientific, Fairlawn, NJ)

urethane (Sigma Chemical Co., St. Louis, MO)

All other chemicals were of reagent grade.

Statistical analyses

The data are expressed as the mean ± the standard error of the mean. Differences between two means were analyzed by two-tailed Student's *t*-test for paired or unpaired data. Differences among 3 (or more) means were analyzed by one- or two-way analysis of variance and either Scheffé's or Duncan's multiple range test. Best fitting lines were determined by linear regression analysis by the method of least squares [Daniel, 1978; Zar, 1974].

The criterion for significance in all experiments was taken as a probability of a chance occurrence in less than 5 times out of 100 ($p < 0.05$).

Calculations were made on a Hewlett-Packard 9815A, Hewlett-Packard 1000, and Texas Instruments-55 calculator.

Solutions

Artifical cerebrospinal fluid [Merlis, 1940; Feldberg et al., 1970]

Solution I:	Sodium chloride	4.050 g	
	Potassium chloride	0.125 g	
	Magnesium chloride·$6H_2O$	0.116 g	
	Sodium phosphate dibasic	0.035 g	
	Sodium bicarbonate	0.880 g	in 200 ml dH_2O
Solution II:	Urea	0.630 g	
	Glucose	0.305 g	in 200 ml dH_2O
Solution III:	Calcium chloride·$2H_2O$	0.093 g	in 50 ml dH_2O

These solutions were stored separately in the refrigerator for up to 3 months. For daily use, the solutions were combined 4:4:1 respectively, and mixed by bubbling with 95% CO_2/5% O_2 for 1 minute. The resultant pH is 7.3-7.5, 10-40 minutes after bubbling.

Phosphate buffered saline

Solution I:	Sodium chloride	9.000 g	
	Sodium phosphate dibasic	1.528 g	in 1 liter dH_2O
Solution II:	Sodium chloride	9.000 g	
	Potassium phosphate monobasic	0.348 g	in 1 liter dH_2O

Solution II (600 - 700 ml) was added to solution I until pH = 7.40.

Substance P radioimmunoassay buffer

Solution I:	Sodium phosphate monobasic dihydrate	15.6 g	in 1 liter dH_2O
Solution II:	Sodium phosphate dibasic	14.2 g	in 1 liter dH_2O

Solution I (about 350 ml) was added to solution II until pH = 7.20. The following compounds were added to 250 ml of this solution;

Sodium chloride	0.73 g
EDTA	0.93 g
Sodium azide	0.05 g
Bovine serum albumin	0.25 g
Phenol Red	2.50 mg

and the pH was readjusted with 10 N sodium hydroxide to 7.2.

Catecholamine radioenzymatic assay [Durrett and Ziegler, 1980]

Stock standards: Norepinephrine, epinephrine, and dopamine were each dissolved in 0.2 N acetic acid, 1 mg/ml, and all three catecholamines were combined, diluted with 0.2 N acetic acid to give a final concentration of 10 µg each in 100 µl. For the standard curve concentrations, 0.01 N hydrochloric acid was used as the diluent.

TEM buffer:	Tris	6.1 g	
	EGTA	1.9 g	
	Magnesium chloride·$6H_2O$	4.7 g	in 250 ml dH_2O

COMT mix:	[^{3}H]S-adenosyl methionine (0.5 mCi/ml)	5 µl
	TEM buffer	84 µl
	O-benzylhydroxylamine (31.9 mg/10 ml)	1 µl
	catechol-O-methyltransferase	10 µl
	glutathione (reduced)	0.06 mg

Borate buffer:	boric acid	11.59 g	
	EDTA	6.25 g	in 125 ml dH_2O

The pH was adjusted to 10 with 10 N sodium hydroxide, and dH_2O was added to a final volume of 250 ml.

Cold carrier:	normetanephrine	120 mg	
	metanephrine	118.6 mg	
	3-methoxytyramine	120 mg	in 100 ml 0.01 N hydrochloric acid

Cold carrier + EtOH/HCl:	normetanephrine	32 mg
	metanephrine	31 mg
	3-methoxytyramine	32 mg
	ethanol	100 ml
	1.0 N hydrochloric acid	10 µl
	dH_2O	5 ml

Toluene:isoamyl alcohol:	2400 ml	toluene
	1600 ml	isoamyl alcohol

Ethylamine solvent:	80 ml	chloroform	
	15 ml	ethanol	
	10 ml	ethylamine	to each chromatography jar

"Phosphor Only":	240 ml	PPO-POPOP
	4000 ml	toluene

Modified Lowry procedure

"Reagent C"	1 ml	potassium sodium tartrate 2%
	1 ml	cupric sulfate 1%
	100 ml	sodium carbonate 2%
"Reagent E"	1 ml	phenol reagent 2 N
	1 ml	dH_2O

RESULTS

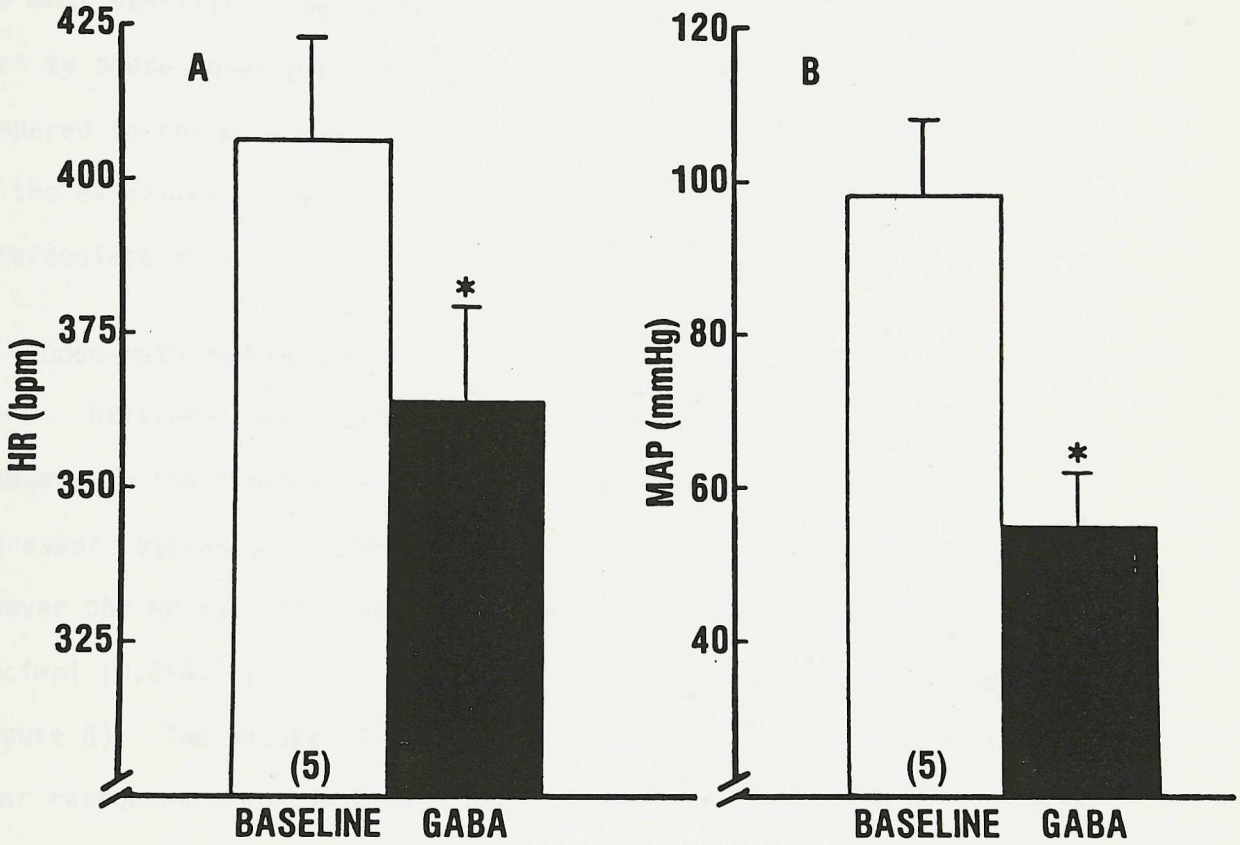
Characterization of GABAergic drug-induced cardiovascular effects at the VSMO in the rat

Initial experiments were done to evaluate the use of a rat model for studying the cardiovascular effects of pharmacologic manipulations of the VSMO. Since the most definitive studies of the cardiovascular responses in the cat involved GABAergic drugs [Feldberg 1976; Guertzenstein et al., 1978; Wennergren and Oberg, 1980; Yamada et al., 1982], the starting point for my work was to use GABAergic drugs to characterize the VSMO in the rat. Specific aims were to: 1) establish the sensitivity and specificity of GABAergic drug-induced cardiovascular responses; 2) localize the effects of GABAergic drugs by monitoring cardiovascular responses to discrete topical application; 3) assess the involvement of the GABAergic drug-induced cardiovascular responses with the autonomic nervous system; 4) evaluate the distribution of [^{3}H]GABA following topical application; 5) determine the importance of the forebrain in mediating GABAergic drug-induced cardiovascular responses.

1. Sensitivity to GABA

To determine whether the ventral surface of the rat's medulla was sensitive to GABA, a dose of 0.234 μmol in 10 μl was injected over the entire exposed ventral surface of 5 rats. Mean heart rate (HR) decreased from 406 ± 17 beats per minute (bpm) to 364 ± 16, a statistically significant change of 42 ± 5 (Figure 3A). Mean arterial pressure (MAP) dropped from 98 ± 10 mmHg to 55 ± 7, a decrease of 43 ± 5 (Figure 3B).

Figure 3. Effects of GABA (0.234 μmol in 10 μl) on heart rate (HR) in beats per minute and mean arterial pressure (MAP) in mmHg when injected over the entire exposed VSMO. ( ) = number of rats. ⊤ = S.E.M. * = $p<0.05$ comparing baselines before and after GABA administration, by Student's *t*-test for paired data.


425
400
375
350
325
HR (bpm)
A
*
(5)
BASELINE
GABA
120
100
80
60
40
MAP (mmHg)
B
*
(5)
BASELINE
GABA

2. Localization of GABA effects

In the next set of experiments I localized the GABA-mediated responses to a more discrete region of the ventral surface. The ventral medulla was divided into seven 0.5 mm^2 areas rostro-caudally and just lateral to the pyramids. The results of placement of GABA-soaked pledgets (0.13 µmol) to these areas from 3 rats is illustrated in Figure 4. The most sensitive areas were the 3 intermediate zones. Drug application to these zones produced twice the magnitude of MAP and HR responses compared to the more rostral and caudal zones. Thus, in the remainder of the experiments the pledgets were enlarged to encompass the entire intermediate area.

3. Dose-response relationships

Bilateral application of GABA (0.023-2.34 µmol) with 1 x 1.5 mm pledgets to the intermediate area produced dose-related bradycardic and depressor responses (Figure 5). The MAP decrease plateaued at 0.78 µmol, however the HR decrease never plateaued. Bilateral application of muscimol (0.044-1.21 nmol) elicited similar decreases in HR and MAP (Figure 6). The onsets of action were immediate and maximal cardiovascular responses occurred about 2 minutes after application. When administered intravenously, these doses of GABA and muscimol produced no changes in HR or MAP.

Equal volumes of i.v. PBS and topically applied CSF to the VSMO did not change MAP or HR or alter the effects of subsequent drug administration in these and subsequent experiments.

4. Interactions of GABA with the autonomic nervous system

The effects of peripherally administered α-adrenoreceptor, β-adrenoreceptor, and muscarinic receptor blocking agents on GABA-induced

Figure 4. Diagram of VSMO showing the range of decreases in heart rate (top) in beats per min (bpm) and mean arterial pressure (bottom) in mmHg due to bilateral pledget (0.5 x 0.5 mm) application of 0.13 µmol GABA (n=3) to the VSMO. The figures show the 7 rostrocaudal levels at which the responses were elicited. Tr (trapezoid body), VI & XII (cranial nerves).

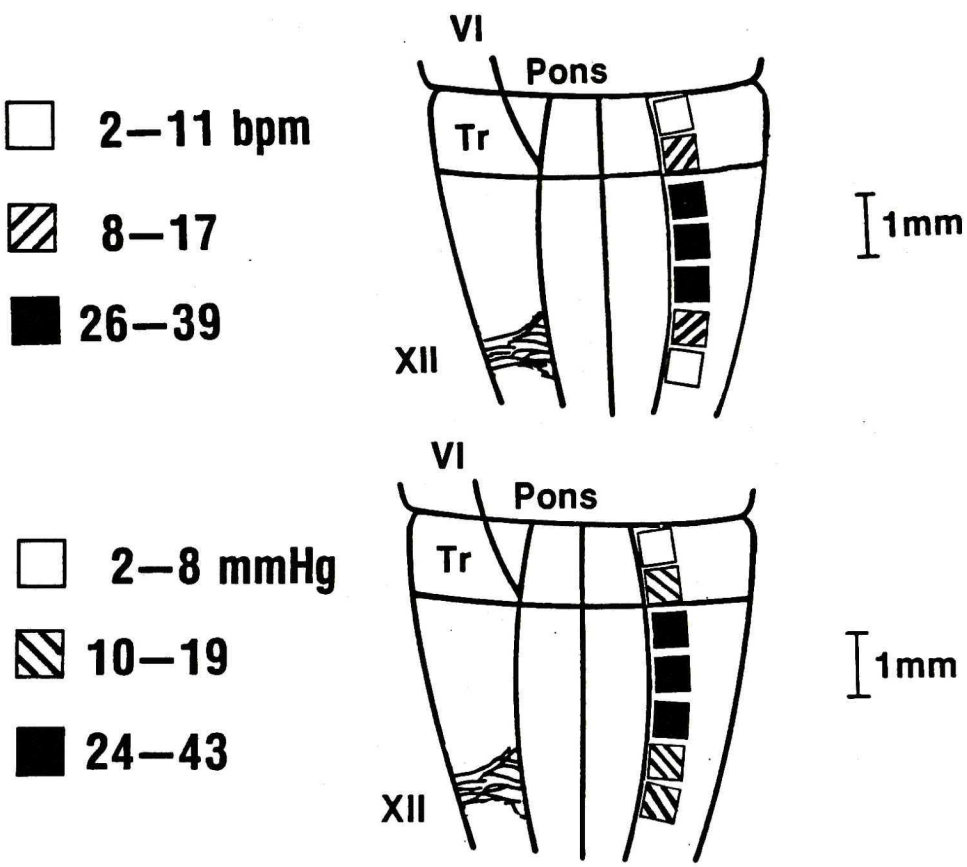
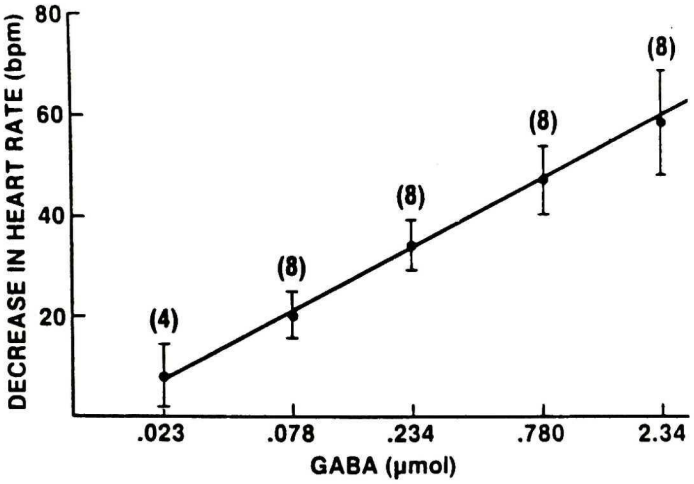

2–11 bpm
8–17
26–39
VI
Pons
Tr
XII
1mm
2–8 mmHg
10–19
24–43
VI
Pons
Tr
XII
1mm

Figure 5. Dose-response relationships of heart rate (top) and mean arterial pressure (bottom) to bilateral pledget application of GABA to the intermediate area of the VSMO. ( ) = number of rats. $\mathbb{I}$ = S.E.M.


DECREASE IN HEART RATE (bpm)
80
60
40
20
(4)
(8)
(8)
(8)
(8)
.023
.078
.234
.780
2.34
GABA (μmol)

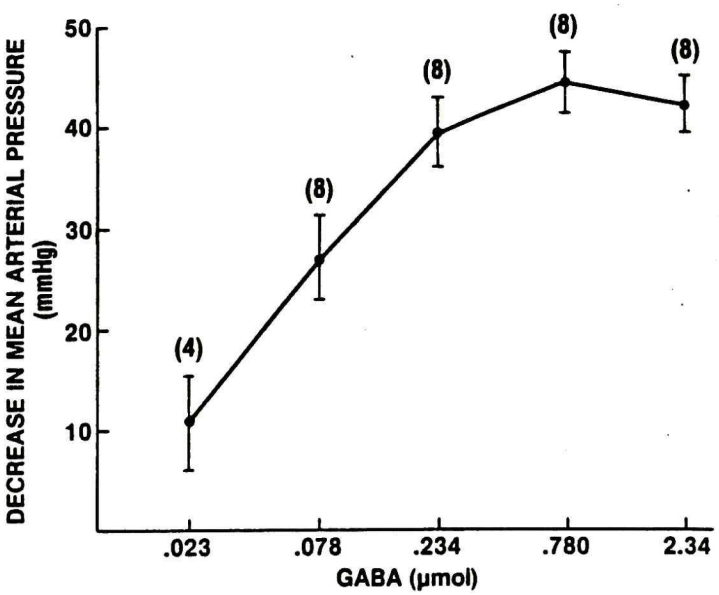
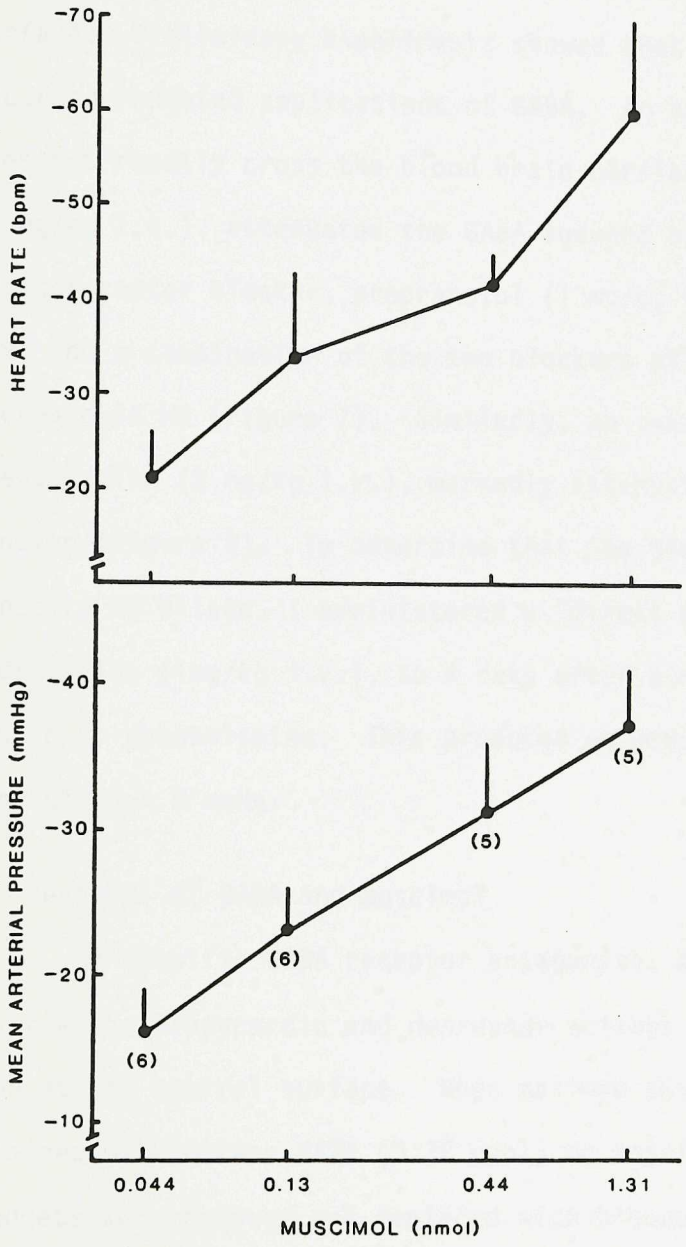

DECREASE IN MEAN ARTERIAL PRESSURE (mmHg)
50
40
30
20
10
(4)
(8)
(8)
(8)
(8)
.023
.078
.234
.780
2.34
GABA (μmol)

Figure 6. Dose-response relationships of heart rate (top) and mean arterial pressure (bottom) to bilateral pledget application of muscimol to the intermediate area of the VSMO. ( ) = number of rats. | = S.E.M.


HEART RATE (bpm)
-70
-60
-50
-40
-30
-20
MEAN ARTERIAL PRESSURE (mmHg)
-40
-30
-20
-10
(6)
(6)
(5)
(5)
0.044
0.13
0.44
1.31
MUSCIMOL (nmol)

(0.78 µmol) bradycardia and hypotension were assessed. In the first part of these experiments, GABA's maximal HR and MAP responses were ascertained. After these parameters returned to control values, the blockers were given intravenously. When the HR and MAP effects of the receptor blocking drugs were maximum, GABA was reapplied to the ventral surface. Preliminary experiments showed that tachyphylaxis did not occur to repeated applications of GABA. An antimuscarinic drug that does not readily cross the blood brain barrier, atropine methylnitrate (1 mg/kg i.v.), attenuated the GABA-induced bradycardia by 37%, a β-adrenoreceptor blocker, propranolol (1 mg/kg i.v.), diminished it by 66%, and a combination of the two blockers effectively eliminated the decrease in HR (Figure 7). Similarly, an α-adrenoreceptor blocker, phentolamine (2 mg/kg i.v.), markedly attenuated the GABA-induced hypotension (Figure 8). To determine that the blood vessels still had the capacity to dilate, I administered a "direct acting" vasodilator, hydralazine (1mg/kg i.v.), to 4 rats after a maximal vasodepressor response to phentolamine. This produced an additional fall in blood pressure of 24 ± 8 mmHg.

5. Reversal of GABA and muscimol

A specific GABA receptor antagonist, bicuculline, was used to reverse the bradycardic and depressor actions of GABA receptor activation at the ventral surface. When maximum decreases in HR and MAP were obtained with either GABA (0.78 µmol) or muscimol (1.31 nmol), the pledgets were removed and replaced with bicuculline methiodide (BMI)-soaked pledgets (0.59 nmol). Return to control values or greater for MAP and HR took 3 and 6-8 minutes, respectively. Results with GABA and muscimol are shown in Figures 9 and 10, respectively. Figure 11 shows a

Figure 7. Effects of GABA (0.78 μmol) topically applied to the intermediate area, on heart rate (HR) in the absence (solid bars) and presence (hatched bars) of autonomic blockers (atropine methylnitrate and/or propranolol ; 1 mg/kg) administered intravenously. Numbers at the base of each histogram are the baseline heart rate (bpm) values prior to topical application of GABA. ( ) = number of rats. ⊥ = S.E.M. * = $\underline{p} < 0.05$ comparing changes from baseline before and after blocker(s), by Student's $\underline{t}$-test for paired data.

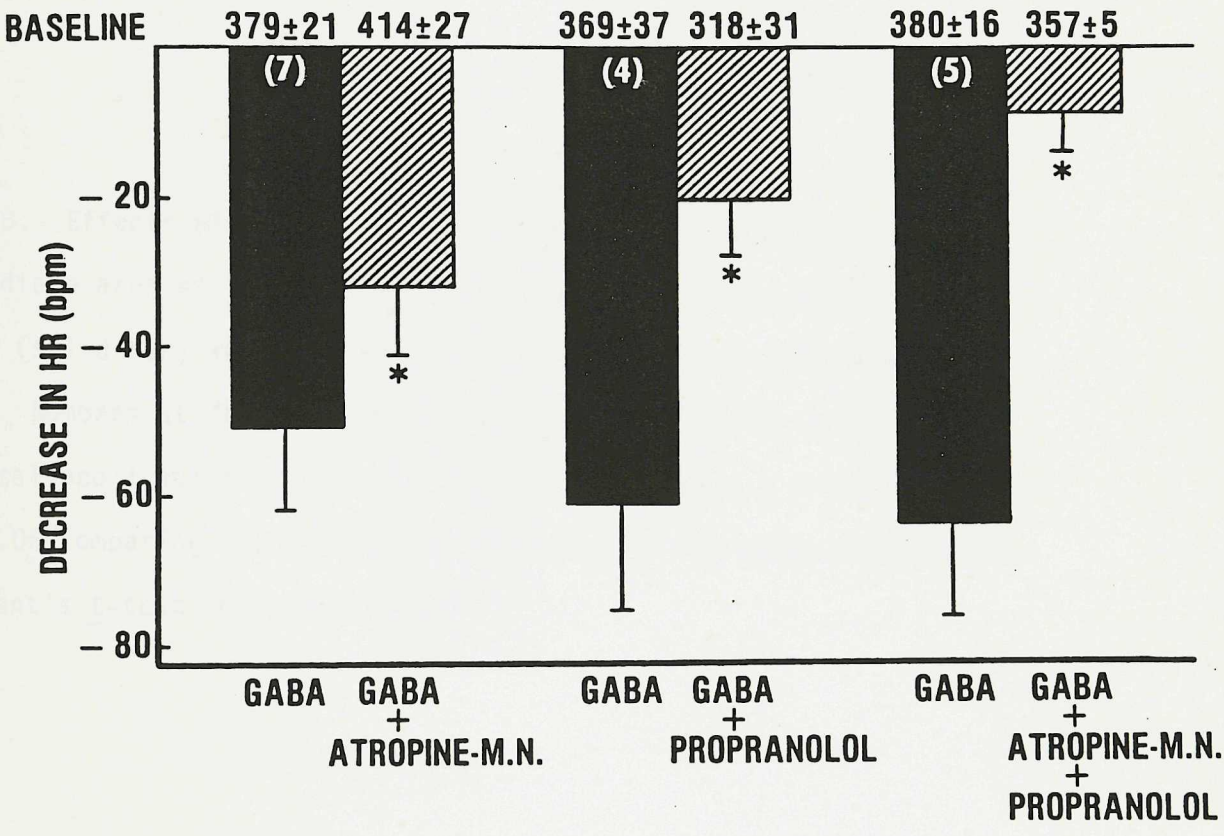

BASELINE
379±21
414±27
369±37
318±31
380±16
357±5
(7)
(4)
(5)
DECREASE IN HR (bpm)
– 20
– 40
– 60
– 80
*
GABA
GABA + ATROPINE-M.N.
GABA + PROPRANOLOL
GABA + ATROPINE-M.N. + PROPRANOLOL

Figure 8. Effects of GABA (0.78 µmol) topically applied to the intermediate area of the VSMO, on mean arterial pressure (MAP) in the absence (solid bar) and presence (hatched bar) of i.v. phentolamine (2 mg/kg). Numbers at the base of each bar are baseline MAP (mmHg) prior to topical application of GABA. ( ) = number of rats. ⊥ = S.E.M. * = $p<0.05$ comparing changes from baseline before and after phentolamine by Student's t-test for paired data.

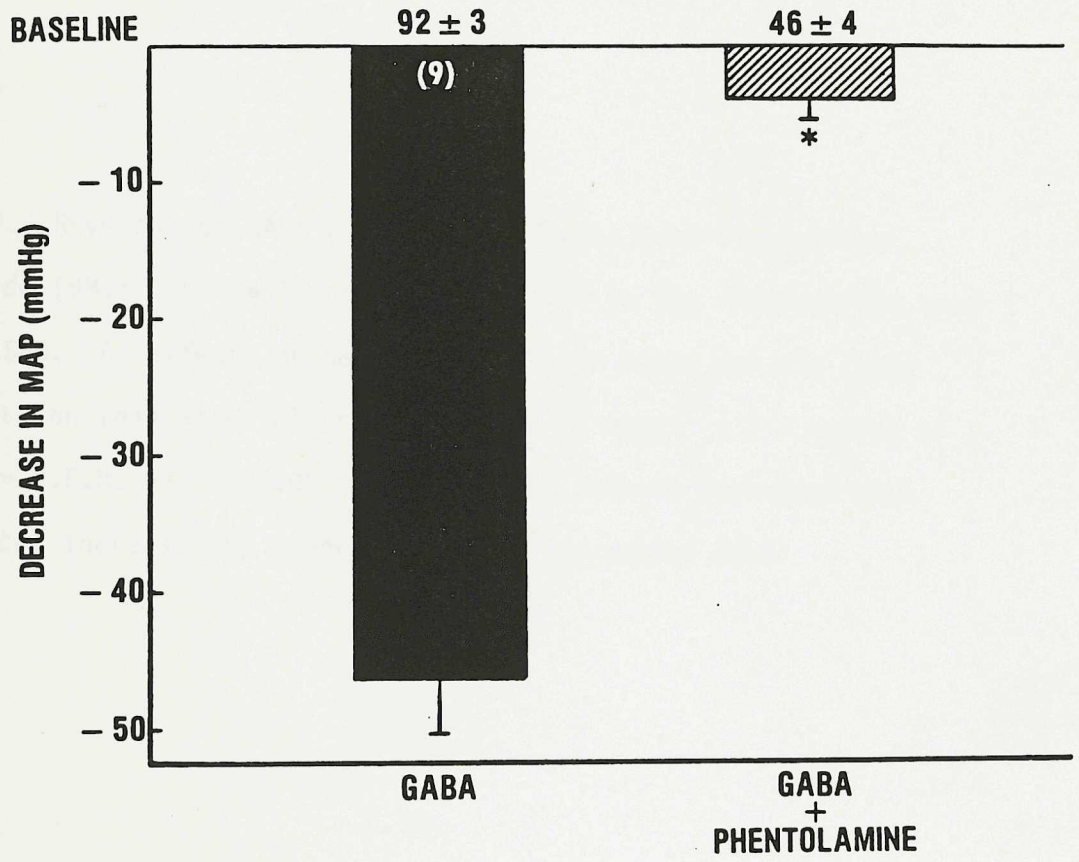

BASELINE
92 ± 3
(9)
46 ± 4
*
– 10
– 20
– 30
– 40
– 50
DECREASE IN MAP (mmHg)
GABA
GABA
+
PHENTOLAMINE

Figure 9. Reversal of GABA (0.78 µmol) effects with bicuculline methiodide (BMI; 0.59 nmol) topically applied to the intermediate area of the VSMO. A: effects on heart rate (HR) in beats per min (bpm) B: effects on mean arterial pressure (MAP) in mmHg. ( ) = number of rats. | = S.E.M. * = $p<0.05$ comparing GABA-induced decreases with BMI-induced increases by Student's *t*-test for paired data.

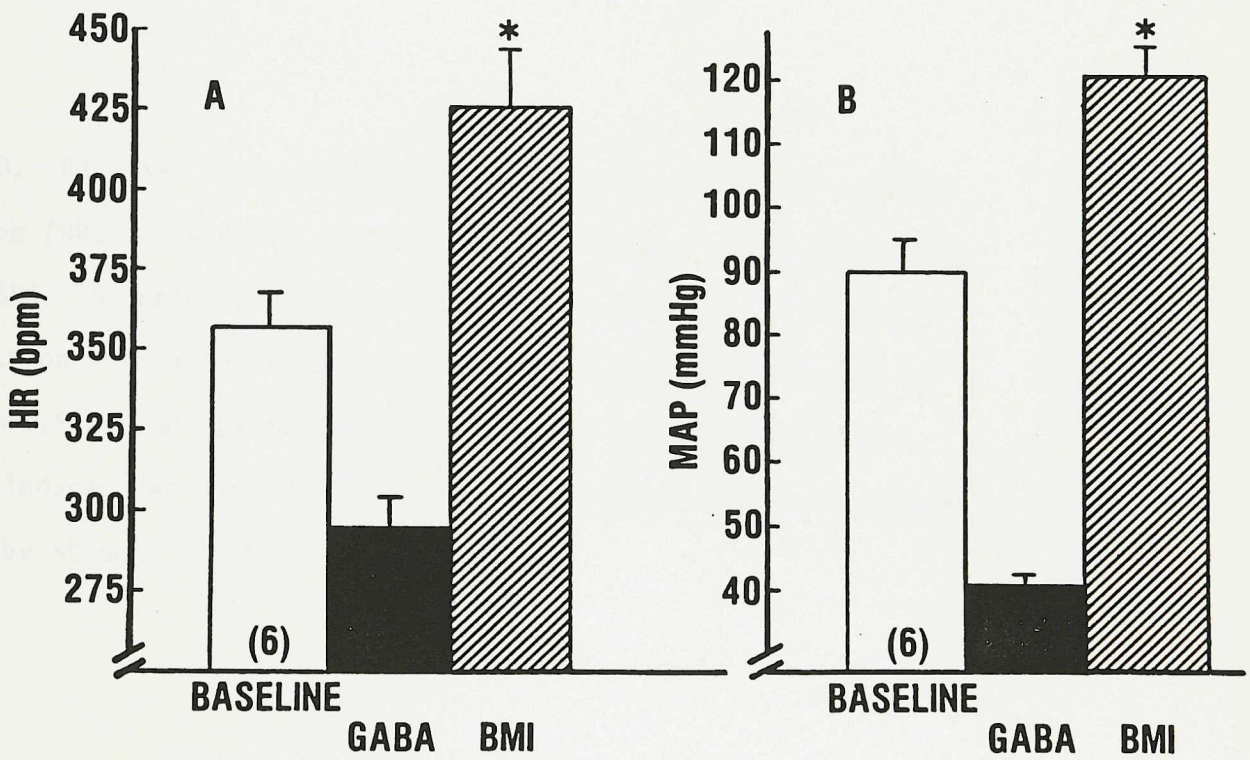

450
425
400
375
350
325
300
275
HR (bpm)
A
*
(6)
BASELINE
GABA
BMI
120
110
100
90
80
70
60
50
40
MAP (mmHg)
B
*
(6)
BASELINE
GABA
BMI

Figure 10. Reversal of muscimol (1.31 nmol) effects with bicuculline methiodide (BMI; 0.59 nmol) topically applied to the intermediate area of the VSMO. A: effects on heart rate (HR) in beats per min (bpm). B: effects on mean arterial pressure (MAP) in mmHg. ( ) = number of rats. | = S.E.M. No statistically significant differences between muscimol-induced decreases and BMI induced increases were observed as measured by Student's t-test for paired data.

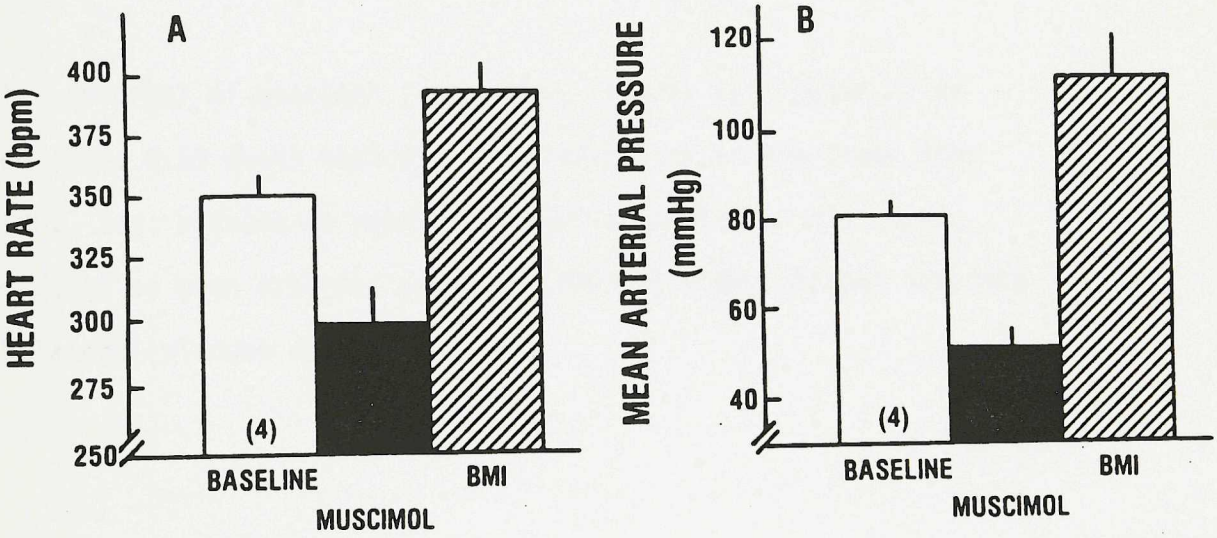
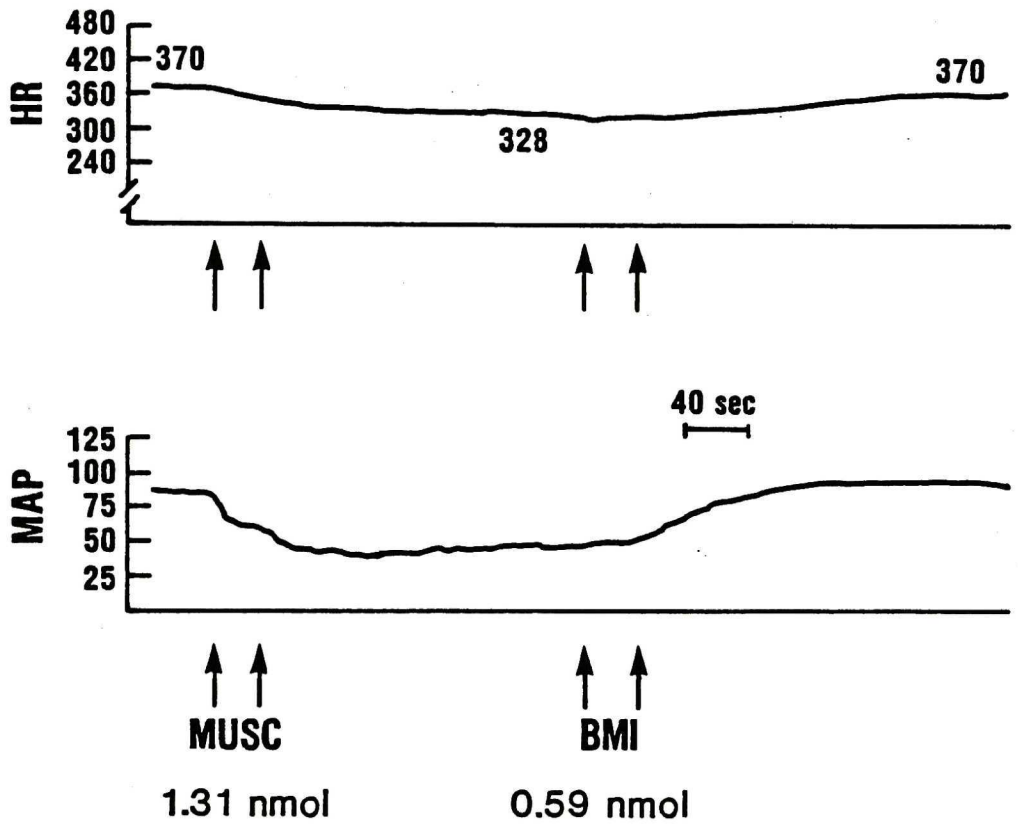

A
HEART RATE (bpm)
400
375
350
325
300
275
250
(4)
BASELINE
BMI
MUSCIMOL
B
MEAN ARTERIAL PRESSURE (mmHg)
120
100
80
60
40
(4)
BASELINE
BMI
MUSCIMOL

Figure 11. Reversal of muscimol (1.31 nmol) effects with bicuculline methiodide (BMI; 0.59 nmol) topically applied to the intermediate area of the VSMO. Top: effects on heart rate (HR) in beats per min (bpm). Bottom: effects on mean arterial pressure (MAP) in mmHg. Arrows indicate time of bilateral pledget application.


480
420
370
360
300
240
HR
370
328
40 sec
125
100
75
50
25
MAP
MUSC
BMI
1.31 nmol
0.59 nmol

representative experiment of the reversal of the muscimol-induced bradycardic and depressor effects with BMI.

The duration of action of GABA and muscimol, after removal of the pledgets, a CSF wash, and allowing spontaneous recovery to control values, was 15-20 minutes. Glycine (0.67 µmol) produced comparable decreases in HR and MAP as GABA (0.78 µmol) at the intermediate area.

To test the specificity of the BMI/GABA interaction, I compared the ability of strychnine (2 nmol) to reverse the cardiovascular effects of glycine and GABA. Strychnine significantly decreased the duration of action of glycine to 48% and 43% of control for HR and MAP, respectively. Strychnine did not alter the magnitude or time-course of the GABA-induced effects (Figure 12).

6. Bicuculline

In the previous experiments, BMI not only reversed the cardiovascular effects of GABA (and muscimol), but also increased MAP and HR to greater than the original baseline levels (Figure 9). This suggested that BMI might be having effects of its own at that site. To test this idea, BMI (0.59 nmol) was applied by pledgets to the intermediate area in the absence of prior drug intervention. The latency of onset was approximately 30 seconds and resulted in a 22% increase in HR that peaked in 6-8 min and a 42% increase in MAP that peaked in 3 minutes (Figure 13). No motor activity was noted.

7. Localization of bicuculline effects

Because the application of BMI to the intermediate area was shown to reverse the cardiovascular effects of GABA and muscimol, and have effects of its own at that site, I examined the sensitivity of BMI

Figure 12. Duration of action in minutes (min), of the bradycardic effects (HR; top) and depressor effects (MAP; bottom), produced by glycine (GLY; 0.67 µmol; solid bars) or GABA (0.78 µmol; cross-hatched bars), applied to the intermediate area of the VSMO. The solid bars show the effect of strychnine (STRYCH; 2 nmol) on the duration of action of the inhibitory amino acids when applied to the intermediate area of the VSMO at the time of their peak effects. ( ) = number of rats. ⊤ = S.E.M. * = $p<0.05$ comparing the durations of action of the inhibitory amino acids with and without strychnine, by Student's t-test for unpaired data.

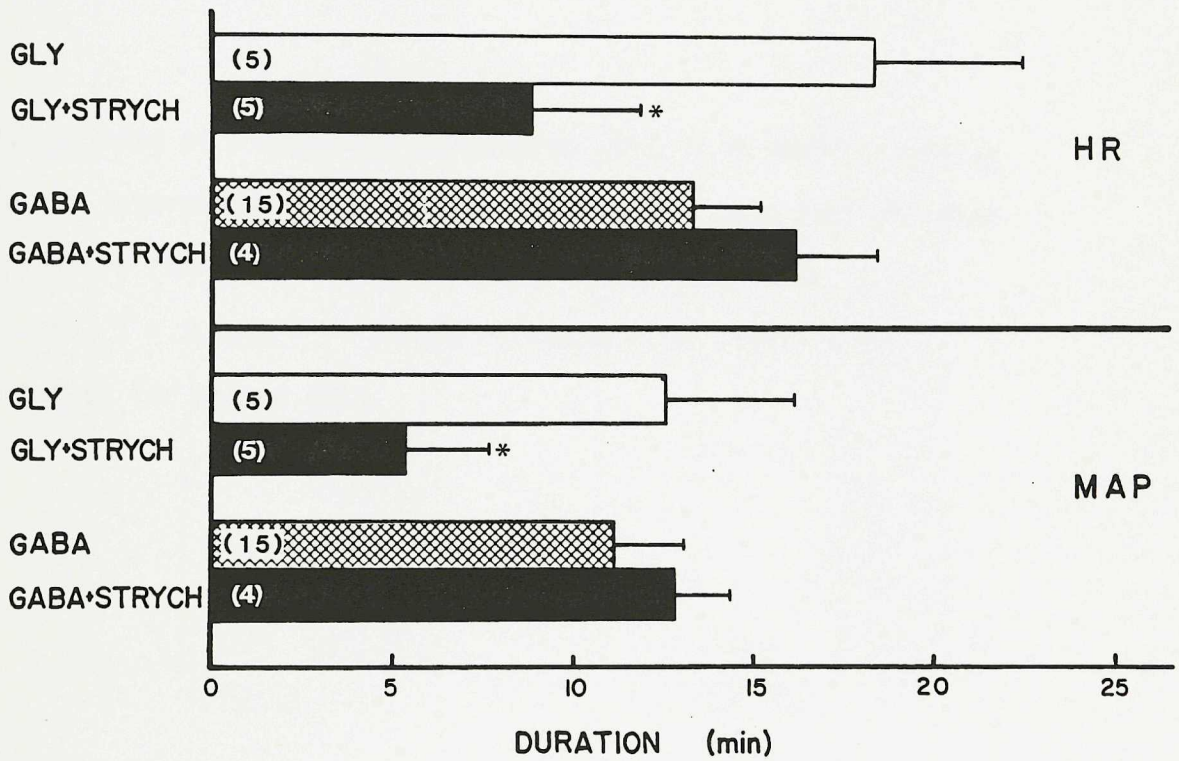
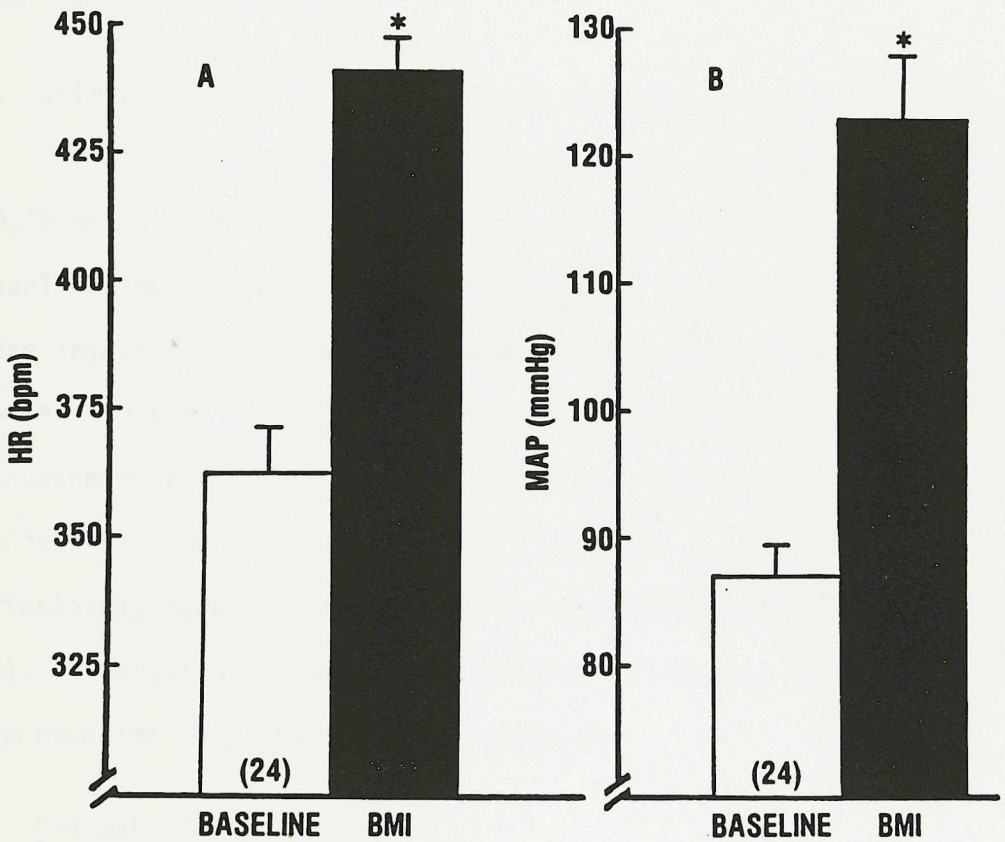

GLY
(5)
GLY+STRYCH
(5)
*
HR
GABA
(15)
GABA+STRYCH
(4)
GLY
(5)
GLY+STRYCH
(5)
*
MAP
GABA
(15)
GABA+STRYCH
(4)
0
5
10
15
20
25
DURATION (min)

Figure 13. Effects of bicuculline methiodide (BMI; 0.59 nmol) topically applied to the intermediate area of the VSMO on heart rate (HR) and mean arterial pressure (MAP) at the intermediate area of the VSMO. ( ) = number of rats. ⊤ = S.E.M. * = $p<0.05$ as compared to baseline by Student's t-test for paired data.


450
425
400
375
350
325
HR (bpm)
A
*
(24)
BASELINE
BMI
130
120
110
100
90
80
MAP (mmHg)
B
*
(24)
BASELINE
BMI

(0.1 nmol) at other sites on the ventral surface. The same protocol was used as for the GABA localization experiments and the results are summarized in the upper part of Figure 14 (the GABA localization results are presented graphically at the bottom of Figure 14 for comparison). The area of greatest sensitivity was found to be mid-way between the rostral trapezoid body and the caudal hypoglossal rootlets. Likewise, for the following BMI experiments I used pledgets that encompassed the 3 small intermediate zones.

8. Interactions of bicuculline with the autonomic nervous system

Initially, I assessed the maximal HR and MAP responses to BMI (0.59 nmol). When the tachycardic and pressor effects had returned to baseline, muscarinic, β-adrenoreceptor, or ganglionic blocking agents were injected intravenously. When the HR and MAP effects produced by the blockers were maximum, BMI was reapplied to the ventral surface. Atropine methylnitrate (1 mg/kg i.v.) had little effect on BMI-induced tachycardia; however, propranolol (1 mg/kg i.v.) or both blockers together effectively reduced the increase in HR normally produced by BMI (Figure 15). A ganglionic blocker, pentolinium (10 mg/kg i.v.), prevented the hypertensive response to BMI (Figure 16).

9. [^{3}H]GABA distribution

The site of action of GABAergic drugs was localized in a rostrocaudal plane to the intermediate area of the VSMO (Figure 14), but it was also important to evaluate their apparent site of action in a dorsoventral plane. I quantitated the amount of tritium in brain tissue after a 2 minute topical application of [^{3}H]GABA, by counting the radioactivity in microdissections of five 300 μm coronal sections (0.5-1 mm

Figure 14. Changes in heart rate (HR) in beats per min (bpm) and mean arterial pressure (MAP) in mmHg due to bilateral pledget (0.5 x 0.5mm) application of 0.13 µmol GABA (n=3), and 0.1 nmol bicuculline methiodide (BMI; n=3), to the VSMO. The inset indicates the 7 levels at which the responses were elicited, and the numbers 1 thru 7 on the bar graph correspond to these levels. HR changes are shown in the dotted bars, and MAP changes in the solid bars. Tr (trapezoid body), VI & XII (cranial nerves). ⊥ ⊤ = S.E.M.

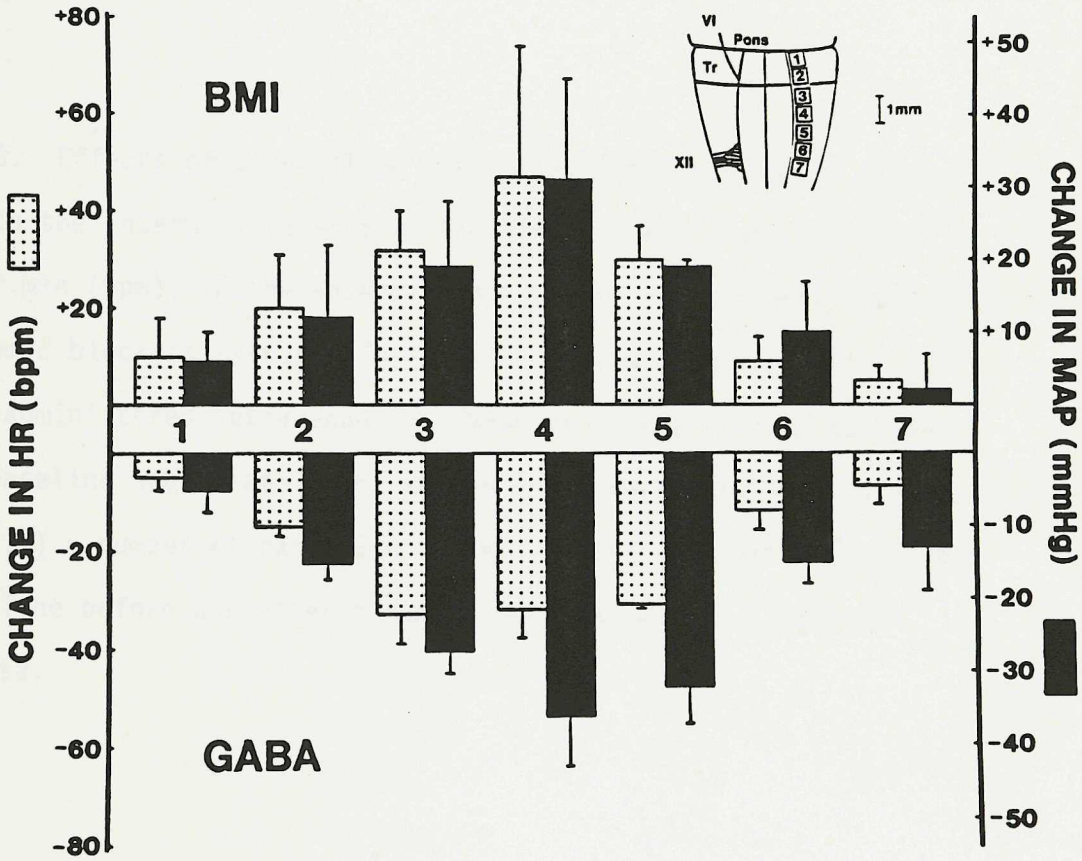

BMI
GABA
CHANGE IN HR (bpm)
CHANGE IN MAP (mmHg)
+80
+60
+40
+20
-20
-40
-60
-80
+50
+40
+30
+20
+10
-10
-20
-30
-40
-50
1
2
3
4
5
6
7
VI
Pons
Tr
XII
1mm

Figure 15. Effects of bicuculline methiodide (BMI; 0.59 nmol) topically applied to the intermediate area of the VSMO on heart rate (HR) in beats per min (bpm), in the absence (empty bars) and presence (solid bars) of autonomic blockers (atropine methylnitrate and/or propranolol; 1 mg/kg) administered intravenously. Numbers at the base of each bar are the baseline heart rate (bpm) values prior to the topical application of BMI. ( ) = number of rats. ⊤ = S.E.M. * = $p<0.05$ comparing changes from baseline before and after blocker(s), by Student's t-test for paired data.

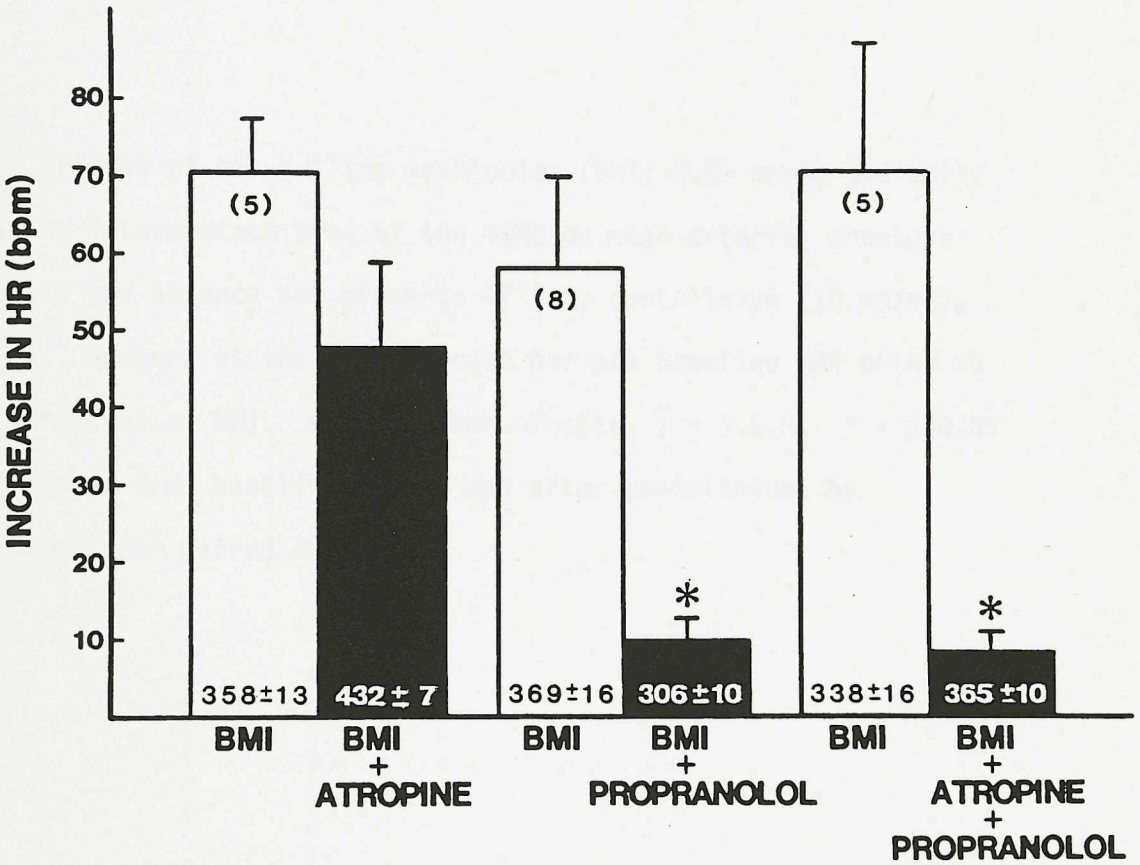
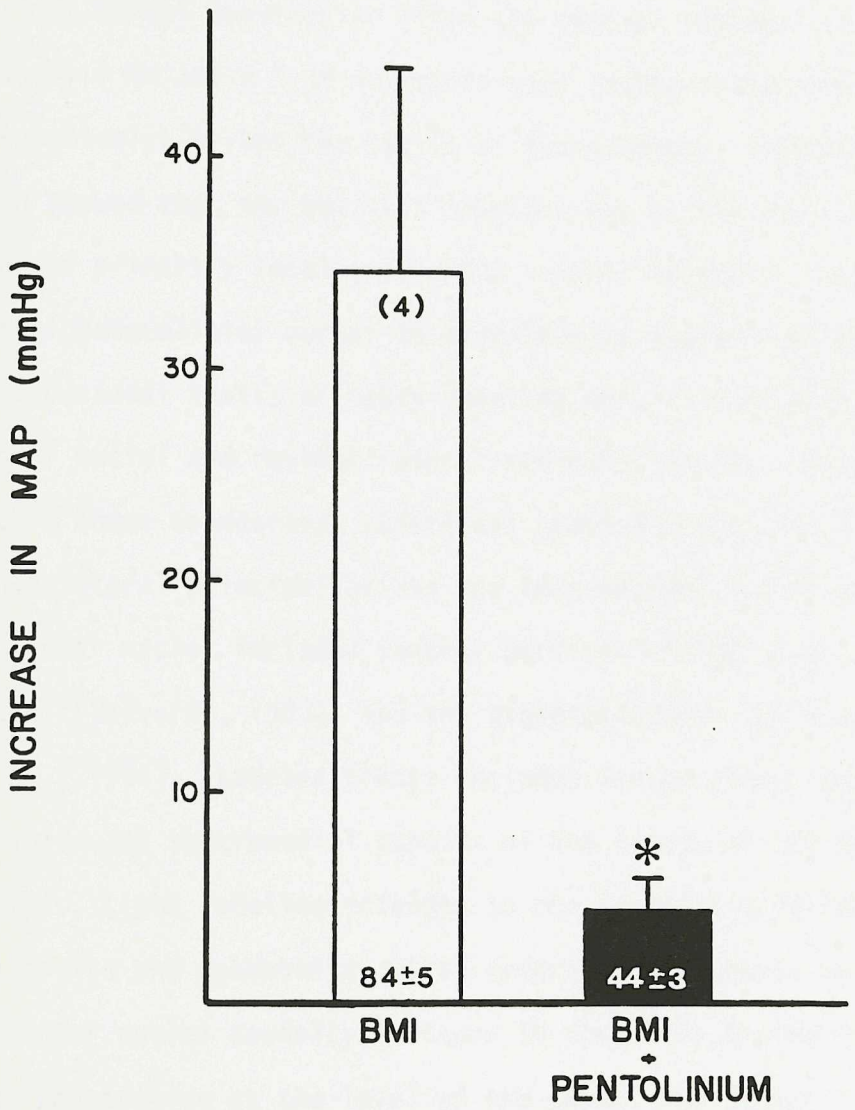

INCREASE IN HR (bpm)
80
70
60
50
40
30
20
10
(5)
(8)
(5)
358±13
432 ± 7
369±16
306±10
338±16
365 ±10
*
*
BMI
BMI + ATROPINE
BMI
BMI + PROPRANOLOL
BMI
BMI + ATROPINE + PROPRANOLOL

Figure 16. Effects of bicuculline methiodide (BMI; 0.59 nmol) topically applied to the intermediate area of the VSMO on mean arterial pressure (MAP; mmHg) in the absence and presence of i.v. pentolinium (10 mg/kg), respectively. Numbers at the base of each bar are baseline MAP prior to topical application of BMI. ( ) = number of rats. ⊤ = S.E.M. * = $p<0.05$ comparing changes from baseline before and after pentolinium, by Student's t-test for paired data.


INCREASE IN MAP (mmHg)
40
30
20
10
(4)
84±5
44±3
*
BMI
BMI
+
PENTOLINIUM

intervals) of the medulla. Of the tritium in these sections, 75% was concentrated in an area between the ventral surface and 0.5 mm dorsally (Figure 17). There was no tritium detected in peripheral blood.

Autoradiography revealed the presence of radioactivity in several nuclei and tracts of the ventral medulla. In general, there was a maximal dorsal penetration (from the ventral surface) of tritum approximately 1 mm and a 0.75 mm superficial rostro-caudal and lateral spread, respectively, beyond the limits of the pledgets. Histological examination showed that the heaviest labeling was at the site of application, and was primarily localized to the ventral halves of the lateral paragigantocellular nuclei as described by Andrezik et al. [1981a]. Rostro-caudal limits of heavy labeling were at the levels of the caudal facial nuclei and rostral lateral reticular nuclei, respectively. Within these boundaries, additional labeled structures included the ventrolateral principal olives and ventromedial facial nuclei. Labeled reticular nuclei included ventral portions of the lateral reticular nuclei [Valverde, 1962], and the gigantocellular (pars α) nuclei [Wunscher et al., 1954]. Labeled tracts included ventrolateral aspects of the pyramids and ventromedial aspects of the tracts of the spinal trigeminal nuclei. Light labeling extended to the superior parolivary nuclei [Palkovits and Jacobowitz, 1974] rostrally and again, to the lateral reticular nuclei caudally. Figure 18 shows the degree of radiolabeled drug penetration at the level of the caudal facial nuclei.

10. Decerebration experiments

To evaluate the contribution of forebrain mechanisms to the GABA- and BMI-induced (at the VSMO) cardiovascular responses, rats were rendered decerebrate, the VSMO was exposed, then GABA or BMI was applied

Figure 17. Schematic diagram of coronal sections of rat's brainstem showing 12 microdissected areas per section. Corresponding levels are shown in the parasaggital diagram in figure 2 (a-e). Numbers are the % total disintegrations per minute counted in these five sections after storing the sections at -70°C that had been previously exposed to topical application of [^{3}H]GABA to the intermediate area of the VSMO for 2 minutes. A dissection of similar size was also taken from the cerebellum (numbers to the right). N = 4 rats.

CEREBELLUM

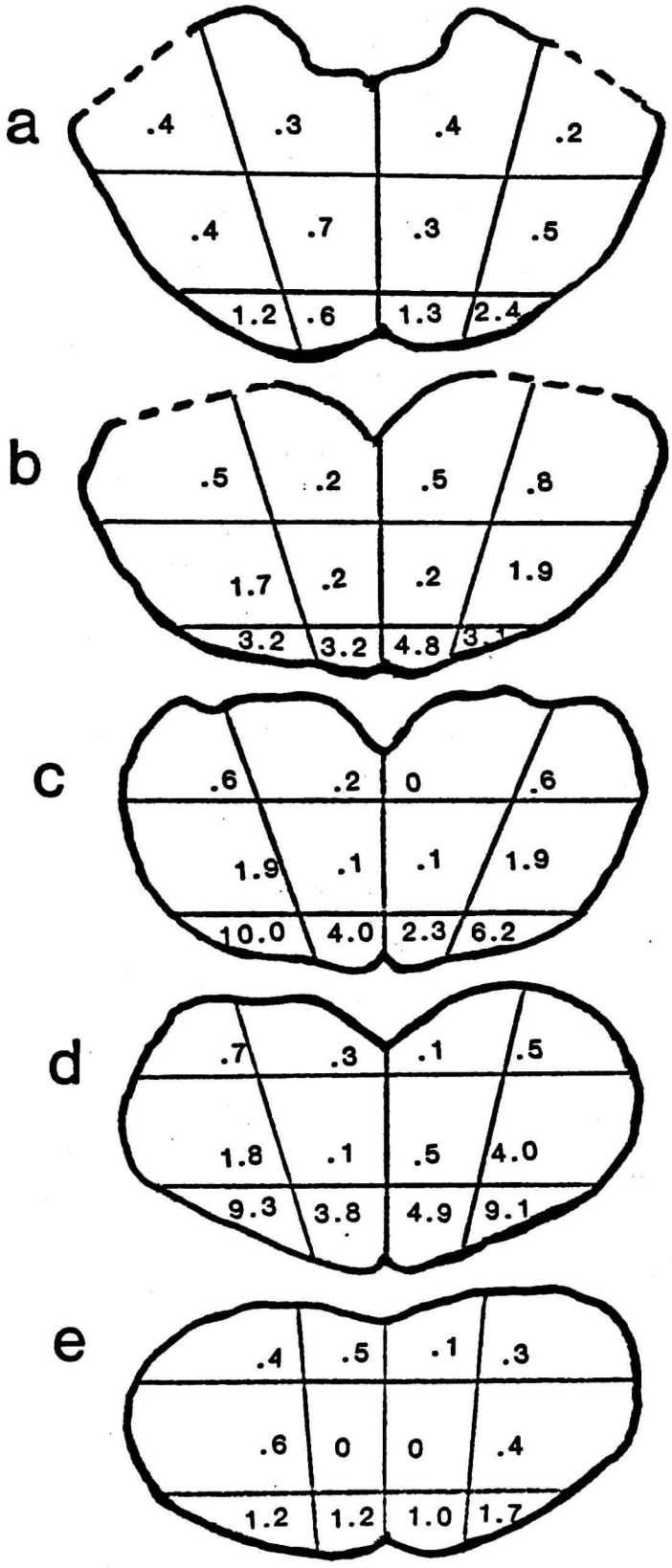
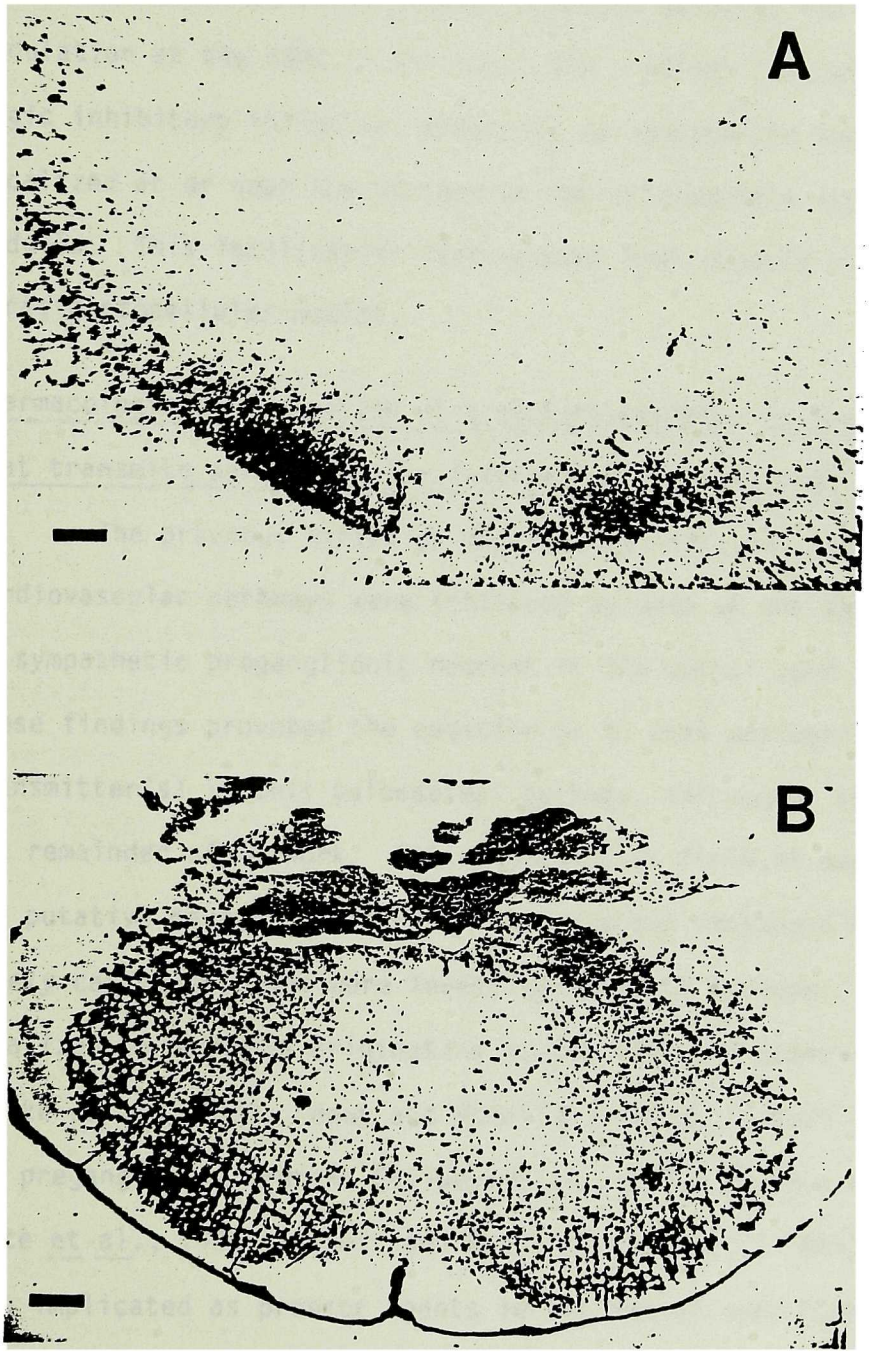

a
.4
.3
.4
.2
.4
.7
.3
.5
1.2
.6
1.3
2.4
.3
b
.5
.2
.5
.8
1.7
.2
.2
1.9
3.2
3.2
4.8
3.1
.1
c
.6
.2
0
.6
1.9
.1
.1
1.9
10.0
4.0
2.3
6.2
.1
d
.7
.3
.1
.5
1.8
.1
.5
4.0
9.3
3.8
4.9
9.1
.1
e
.4
.5
.1
.3
.6
0
0
.4
1.2
1.2
1.0
1.7
.1

Figure 18. A: autoradiogram of 20 μm coronal section showing penetration of [^{3}H]GABA applied to the intermediate area of the VSMO. B: thionin-stained adjacent 20 μm section. Bar = 500 μm.


A
B

to the intermediate area. There were no differences in MAP or HR responses between the decerebrate and sham-operated rats. (Figure 19).

In summary, these studies provided evidence for cardiovascular modulation at the VSMO in the rat. The findings indicated a tonic GABAergic inhibitory influence, primarily on sympathetic outflow, which was localized at or near the surface at the intermediate area of the ventral medulla. This localization corresponded most closely with the lateral paragigantocellular nuclei.

Pharmacologic determination of a neurotransmitter in the spinal cord that transmits cardiovascular information from the VSMO to the IML

The previous series of experiments indicated that excitatory cardiovascular pathways were inhibited by GABA at the VSMO and projected to sympathetic preganglionic neurons in the spinal cord (Figure 20). These findings provoked the question as to what was(were) the neurotransmitter(s) in this bulbospinal pathway, and became the basis for the remainder of my work. Out of the entire field of neurotransmitters and putative neurotransmitters, serotonin and substance P were two likely candidates, and were investigated first because: 1) both were localized to pathways originating at the VSMO and terminating in the IML [Helke et al., 1982; Loewy and McKellar, 1981]; 2) both excited sympathetic preganglionic neurons [Gilbey et al., 1983; Backman and Henry, 1984; Coote et al., 1981; deGroat and Ryall 1967; McCall, 1983]; and 3) both were implicated as pressor agents in the spinal cord [Loewy and Sawyer, 1982; Howe et al., 1983b]. Specific aims were to: 1) determine a possible neurotransmitter mediator of the GABAergic drug-induced effects at the

Figure 19. Effects of bicuculline methiodide (BMI; 0.59 nmol; top) and GABA (0.78 µmol; bottom) on changes in mean arterial pressure (MAP; mmHg) and heart rate (HR; bpm) from topical application to the intermediate area of the VSMO, in decerebrate (solid bars) and sham operated rats (open bars). Numbers at the base of bars are baseline MAP or HR values before application of BMI or GABA. | = S.E.M. No statistically significant differences in the BMI or GABA responses were observed between decerebrate and sham operated rats was observed as measured by Student's t-test for unpaired data.

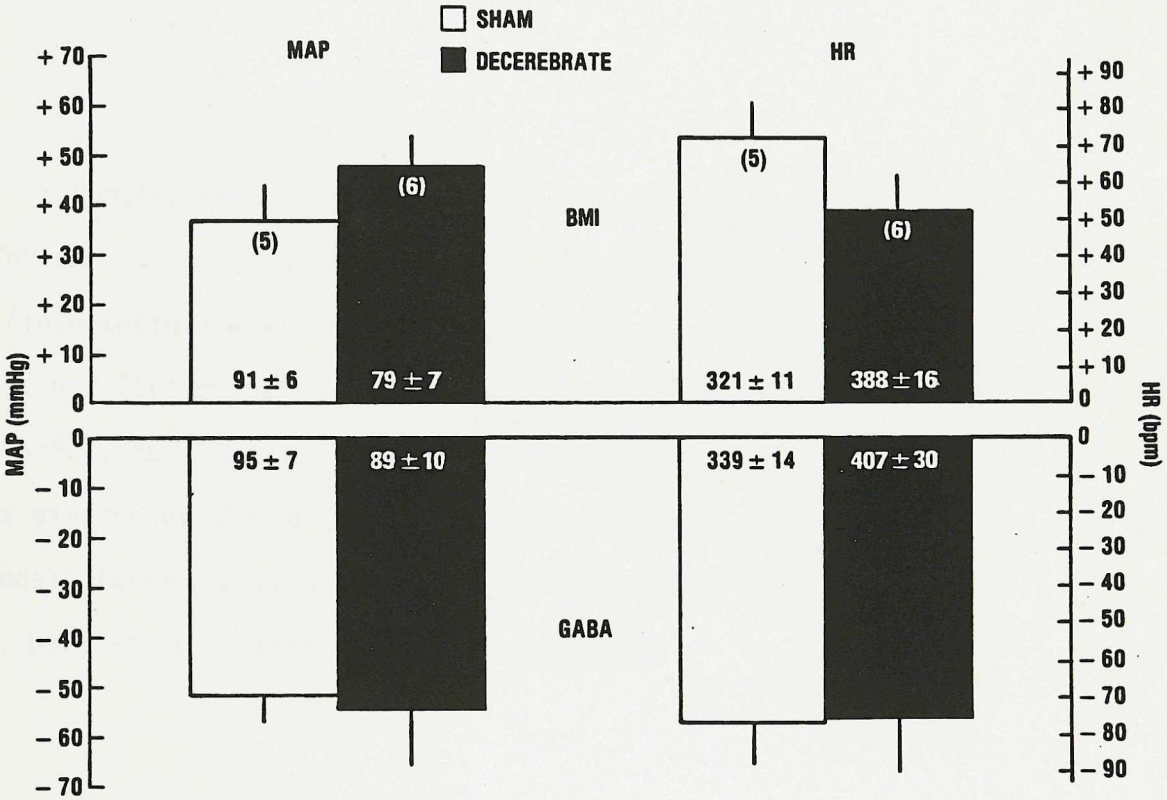
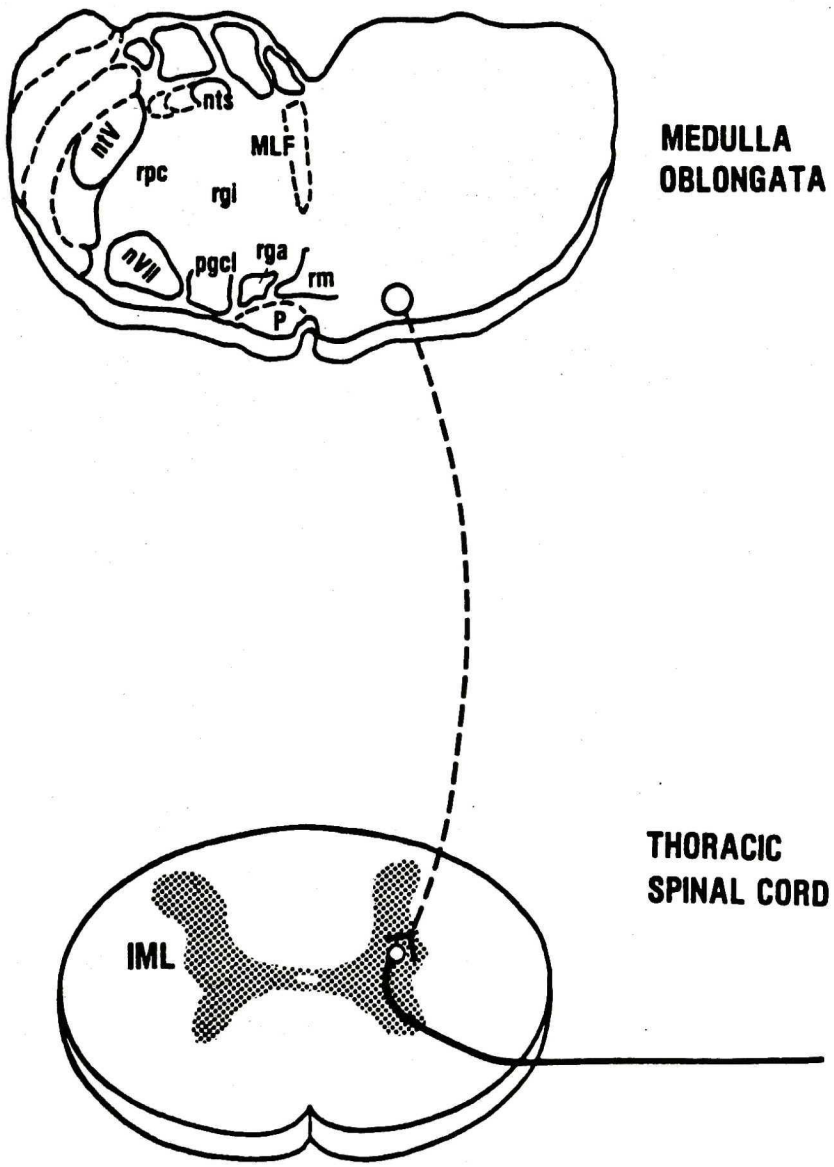

SHAM
DECEREBRATE
MAP
HR
BMI
GABA
MAP (mmHg)
HR (bpm)
+ 70
+ 60
+ 50
+ 40
+ 30
+ 20
+ 10
0
– 10
– 20
– 30
– 40
– 50
– 60
– 70
+ 90
+ 80
+ 70
+ 60
+ 50
+ 40
+ 30
+ 20
+ 10
0
– 10
– 20
– 30
– 40
– 50
– 60
– 70
– 80
– 90
(5)
(6)
(5)
(6)
91 ± 6
79 ± 7
321 ± 11
388 ± 16
95 ± 7
89 ± 10
339 ± 14
407 ± 30

Figure 20. Schematic coronal illustrations of a descending projection from the intermediate area of the ventral surface of the medulla to the IML. IML (intermediolateral cell column), nts (nucleus tractus solitarius), ntV (trigeminal nucleus), rpc (nucleus reticularis parvocellularis), MLF (medial longitudinal fasciculus), rgi (nucleus reticularis gigantocellularis), nVII (facial nucleus), pgcl (lateral paragigantocellularis nucleus), rga (nucleus reticularis gigantocellularis, pars α), rm (raphe magnus), P (pyramid).


nts
nV
MLF
rpc
rgi
nVII
pgcl
rga
rm
P
MEDULLA
OBLONGATA
IML
THORACIC
SPINAL CORD

VSMO, by assessing the effects on the cardiovascular system of an intrathecally injected antagonist to that neurotransmitter; 2) study the connection with the VSMO by attempting to block activation of VSMO pathways with a neurotransmitter antagonist injected intrathecally; 3) verify that the cardiovascular responses were due to the inferred neurotransmitter by injecting the appropriate agonist and monitoring those responses; 4) verify the connection with the IML by attempting to block the agonist's actions with peripherally administered sympathetic blockers.

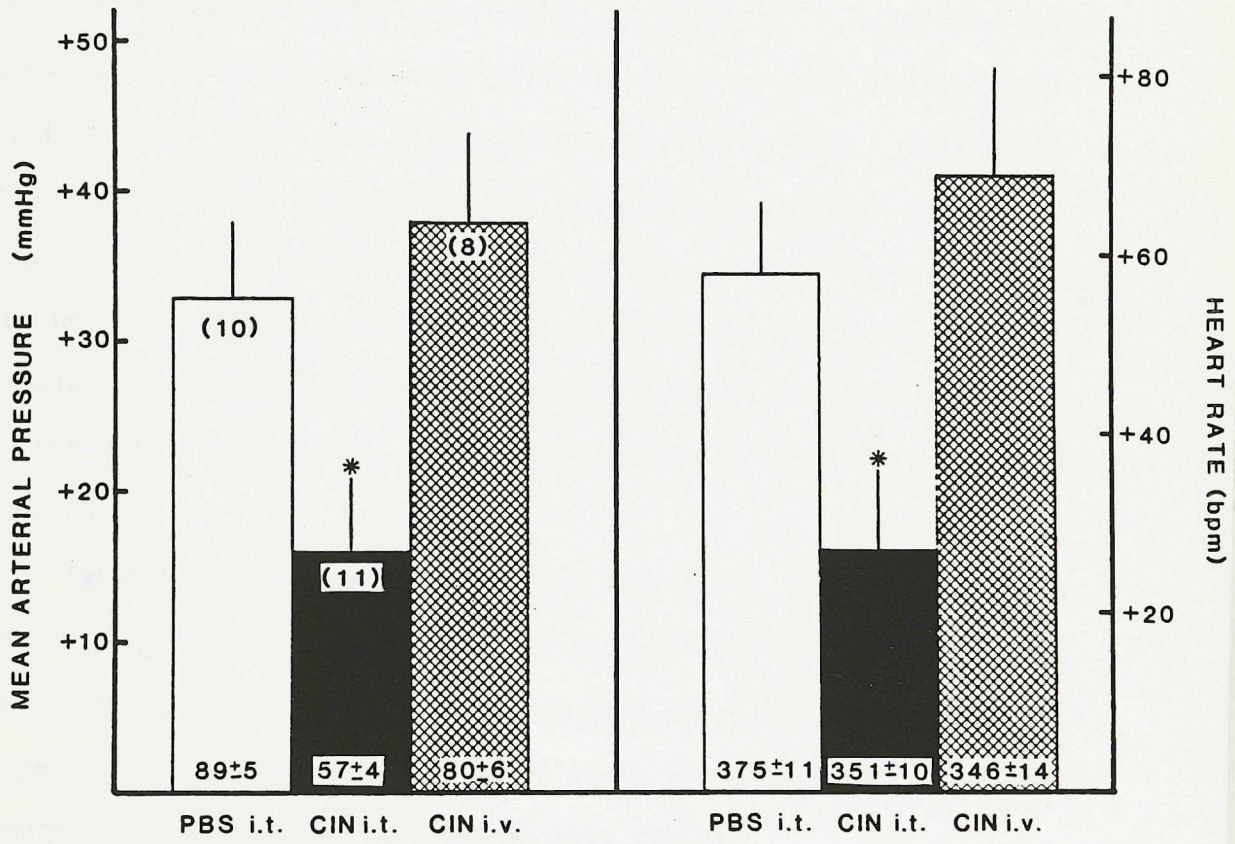
1. Cinanserin

Cinanserin, a 5-HT receptor blocker, decreased the MAP and HR, but did not block the responses to BMI (0.59 nmol) topically applied to the intermediate area of the VSMO, when injected i.t. (150 µg; 398 nmol). A higher dose (350 µg; 928 nmol i.t.) had equally depressant effects on HR whether given i.v. or i.t. (perhaps due to leakage into the systemic circulation), but depressed MAP only when given i.t. The MAP and HR responses to BMI were reduced by 50% after cinanserin 350 µg i.t. (as compared to BMI after cinanserin i.v., PBS i.t., or BMI alone from previous studies (Figure 21).

2. Methysergide

To further evaluate the role of spinal cord 5-HT in these cardiovascular responses, another 5-HT receptor blocker was injected i.t. in three rats (not illustrated). Methysergide (64-340 nmol i.t.) increased the heart rate (+ 20 ± 6 bpm) and decreased the MAP (- 20 ± 2 mmHg). There were no consistent effects when administered i.v. Higher doses (170 and 340 nmol) had to be dissolved in 35-65% methanol to go into solution, but the responses to BMI (0.59 nmol) at the VSMO were not

Figure 21. Increases in mean arterial pressure (left) and heart rate (right) produced by bicuculline methiodide (BMI; 0.59 nmol) applied to the intermediate area of the VSMO when preceded by phosphate buffered saline (PBS 15 µl) i.t. (open bars). cinanserin 350 mg (928 nmol) i.t. (solid bars), or cinanserin 350 mg i.v. (cross-hatched bars). Numbers at the bottom of bars are baseline mean arterial pressure or heart rate values. ( ) = number of rats. | = S.E.M. * = $p<0.05$ compared to PBS i.t., † = $p<0.05$ compared to cinanserin i.v., by 1-way ANOVA and Duncan's multiple range test.


MEAN ARTERIAL PRESSURE (mmHg)
+50
+40
+30
+20
+10
HEART RATE (bpm)
+80
+60
+40
+20
(10)
(11)
(8)
*
*
89±5
57±4
80±6
375±11
351±10
346±14
PBS i.t.
CIN i.t.
CIN i.v.
PBS i.t.
CIN i.t.
CIN i.v.

blocked at any dose.

3. 5,7-dihydroxytryptamine (5,7-DHT)

Since the results from the cinanserin and methysergide experiments were inconsistent, and these 5-HT receptor antagonists are known to bind to non-5-HT receptors in the CNS [Leysen et al., 1981], I tried a more specific approach to clarify these disparities. The serotonin neurotoxin, 5,7-DHT (200 µg x 2), was injected i.t. (45 minutes after desmethylimipramine 25 mg/kg i.p. to prevent uptake of 5,7-DHT into catecholamine nerve terminals) to decrease the level of 5-HT in the spinal cord. Two weeks later, the in vivo experiments were done. Intrathecal injections of 5,7-DHT resulted in 56% depletion of serotonin in the thoracic spinal cord (Figure 22). This depletion did not modify baseline MAP or HR. In addition, serotonin depletion did not change the normal cardiovascular responses to BMI (MAP + 30 $\pm$ 2 mmHg; HR + 68 $\pm$ 13 bpm) or GABA (MAP - 48 $\pm$ 5 mmHg; HR - 72 $\pm$ 7 bpm) topically applied to the VSMO (Figure 23).

Taken together, the data from the cinanserin, methysergide, and 5,7-DHT experiments suggested that 5-HT was probably not a neurotransmitter in the excitatory neuronal system that was inhibited by GABA at the VSMO. These results prompted me to evaluate other spinal cord neurotransmitters that might be involved in this system.

4. Substance P antagonists

Because of the discrepant 5-HT data, I proceeded to examine the potential role of another neurotransmitter candidate in the spinal cord, SP, in these cardiovascular events. The effects of putative SP antagonists injected i.t. were compared with the blood pressure and heart rate

Figure 22. Serotonin content (ng/g frozen tissue weight) in the thoracic spinal cord. Cross-hatched bar: 5,7-dihydroxytryptamine-treated (45 minutes after injection of desmethylimipramine, 1ml/mg) rats (200 µg i.t. x 2). Open bar: Vehicle-treated rats. ( ) = number of rats. | = S.E.M. * = $p<0.05$ by Student's *t*-test for unpaired data.

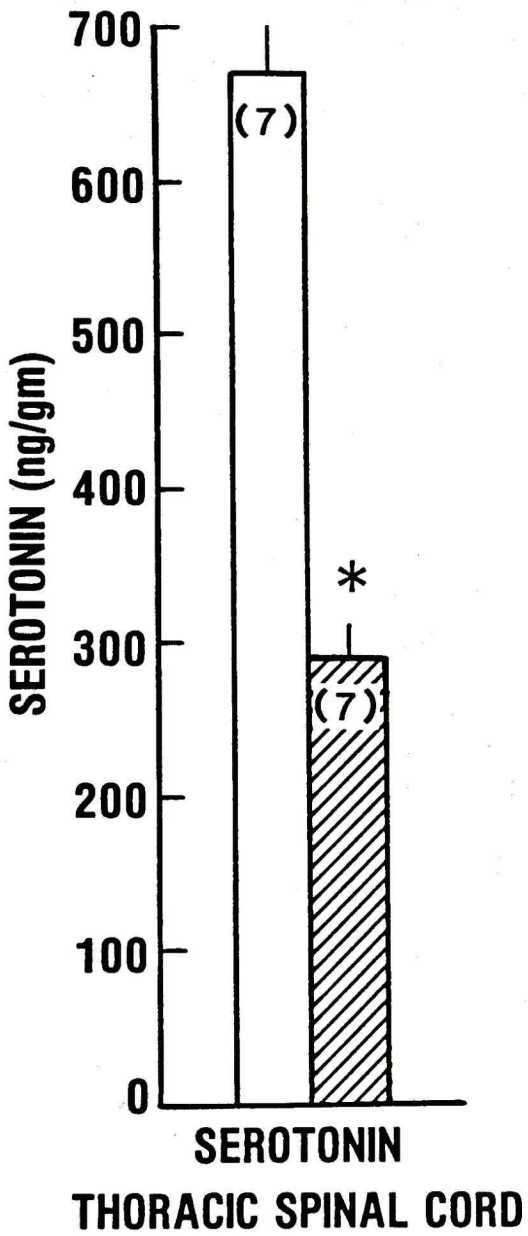
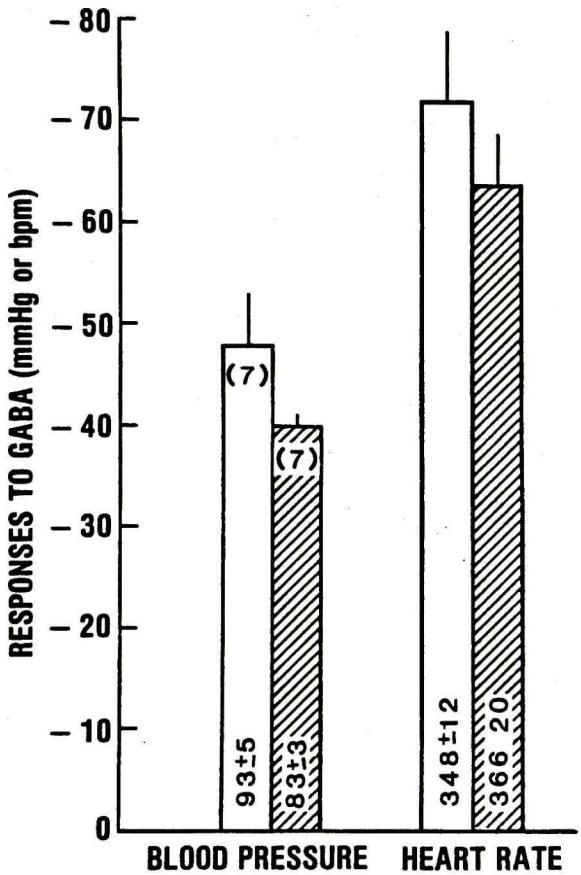
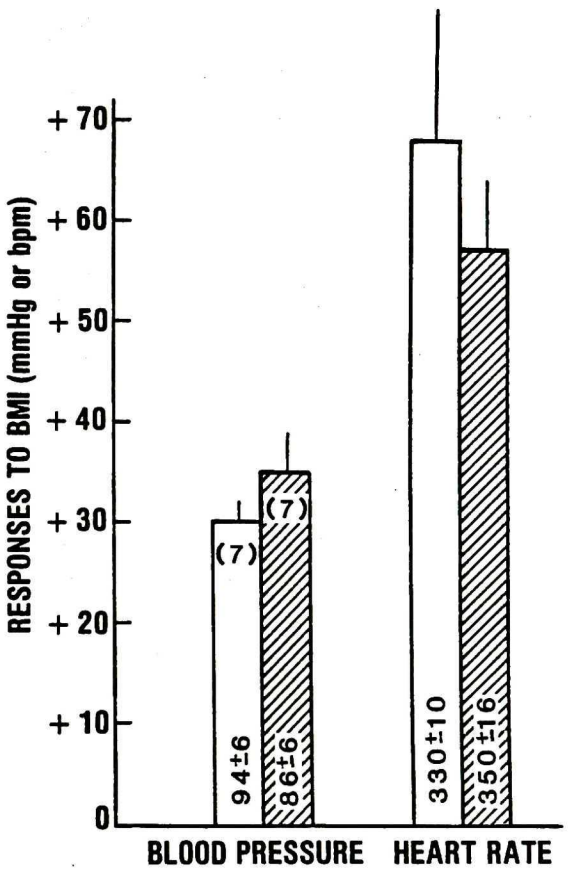

700
600
500
400
300
200
100
0
SEROTONIN (ng/gm)
(7)
*
(7)
SEROTONIN
THORACIC SPINAL CORD

Figure 23. Cardiovascular responses to GABA (0.78 µmol) or bicuculline methiodide (BMI; 0.59 nmol) applied to the intermediate area of the VSMO. Open bars: Vehicle-treated rats. Cross-hatched bars: 5,7-dihydroxytryptamine-treated rats (200 µg i.t. x2). ( ) = number of rats in each group. Numbers in bars are baseline values. | = S.E.M. * = $p<0.05$ by Student's *t*-test for unpaired data.


RESPONSES TO GABA (mmHg or bpm)
− 80
− 70
− 60
− 50
− 40
− 30
− 20
− 10
0
(7)
(7)
93±5
83±3
348±12
366 20
BLOOD PRESSURE
HEART RATE


RESPONSES TO BMI (mmHg or bpm)
+ 70
+ 60
+ 50
+ 40
+ 30
+ 20
+ 10
0
(7)
(7)
94±6
86±6
330±10
350±16
BLOOD PRESSURE
HEART RATE

effects of PBS injected i.t. and SP antagonists injected i.v. Initially, I injected 50 µg of each antagonist in order to compare the effects of the four antagonists, [D-Pro^2, D-Trp^{7,9}]-SP "antagonist I", [D-Pro^2, D-Phe^7, D-Trp^9]-SP "antagonist II", [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-SP "antagonist III", but not [D-Pro^4, D-Trp^{7,9,10}]-SP(4-11) "antagonist IV", caused a long lasting (more than 2 1/2 hours) decrease in MAP (Figure 24). Immediate and short lasting depressor and pressor effects were followed by the gradual depressor phase that reached its nadir, approximately 2/3 baseline values, in 20 minutes. SP antagonists I-III did not change HR. SP antagonist IV increased HR (Figure 25).

Bicuculline methiodide (BMI) applied to the VSMO caused typical increases in MAP (+ 35-45 mmHg) and HR (+ 49-97 bpm) that peaked in 3-8 minutes and lasted up to one hour. When BMI was applied to the VSMO 20 minutes after i.t. injection of SP antagonists (50 µg), the BMI-induced increases in MAP and HR were blocked by each of the SP antagonists except antagonist IV (Figures 26,27). Maximal attenuation of the BMI-induced responses was to 16% and 11% of control MAP and HR responses, respectively. The BMI responses were not blocked by i.t. injection of PBS or i.v. injection of SP antagonists. A representative experiment is shown in Figure 28.

A lower dose (5 µg or 3.3 nmol i.t.) of a SP antagonist (III) also effectively blocked the cardiovascular effects of BMI application to the VSMO. Maximal blockade of MAP (Figure 29) and HR (Figure 30) was measured at 20 minutes. MAP and HR responses to BMI returned to baseline values in one and two hours, respectively.

5. Capsaicin experiments

Although, the previous data supported the concept that spinal

Figure 24. Effects of SP antagonists (50 mg i.t.) on mean arterial pressure 20 min after injection. Open bars: phosphate buffered saline (PBS 15 µl) injected i.t. Cross-hatched bars: SP antagonist injected i.t. Dotted bars: SP antagonist injected i.v. I = D-Pro^2, D-Trp^{7,9}-SP. II = D-Pro^2, D-Phe^7, D-Trp^9-SP. III = D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}-SP. IV = D-Pro^4, D-Trp^{7,9,10}-SP(4-11). N = 5-6 rats in each group. | = S.E.M. a = $p<0.05$ comparing PBS i.t. and SP antagonist i.t., b = $p<0.05$ comparing SP antagonist i.t. and SP antagonist i.v., by 1-way ANOVA and Scheffé's multiple comparison test.

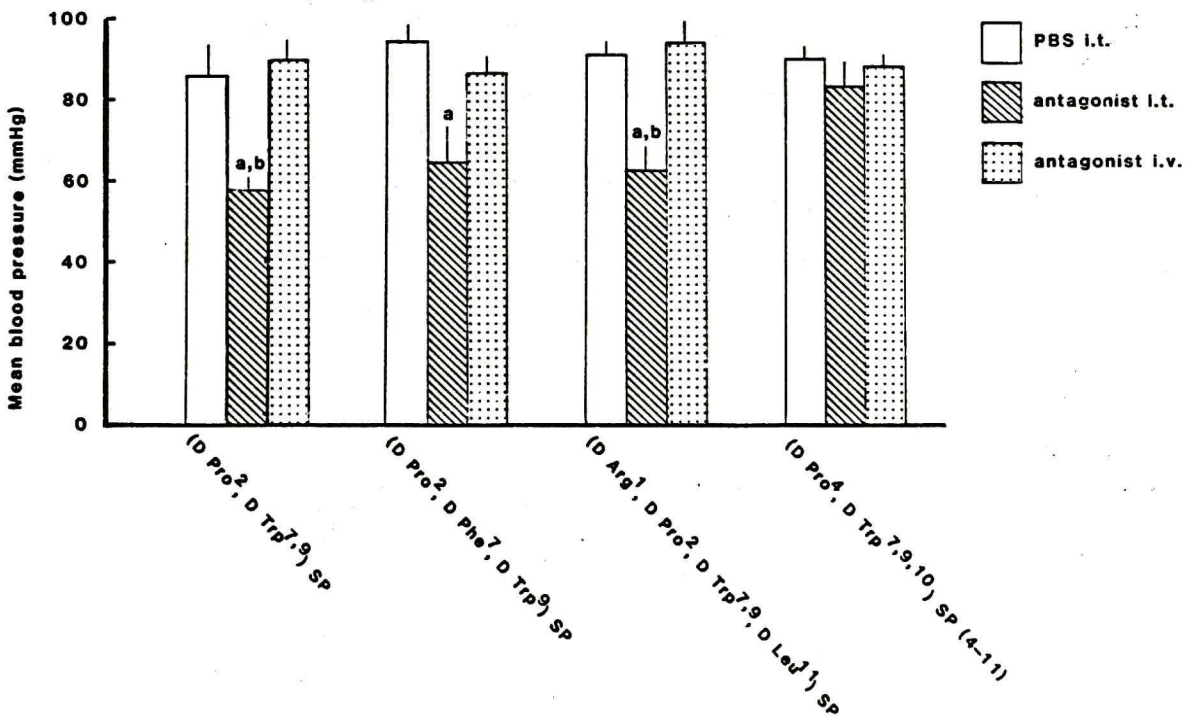

Mean blood pressure (mmHg)
100
80
60
40
20
0
a,b
a
a,b
PBS i.t.
antagonist i.t.
antagonist i.v.
(D Pro2, D Trp7,9) SP
(D Pro2, D Phe7, D Trp9) SP
(D Arg1, D Pro2, D Trp7,9, D Leu11) SP
(D Pro4, D Trp 7,9,10) SP (4-11)

Figure 25. Effects of SP antagonists (50 mg i.t.) on heart rate 20 minutes after injection. Open bars: phosphate buffered saline (PBS 15 µl) injected i.t. Cross-hatched bars: SP antagonist injected i.t. Dotted bars: SP antagonist injected i.v. I = D-Pro^{2}, D-Trp^{7,9}-SP. II = D-Pro^{2}, D-Phe^{7}, D-Trp^{9}-SP. III = D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}-SP. IV = D-Pro^{4}, D-Trp^{7,9,10}-SP(4-11). N = 5-6 rats in each group. | = S.E.M. a = $p<0.05$ comparing PBS i.t. and SP antagonist i.t., b = $p<0.05$ comparing SP antagonist i.t. and SP antagonist i.v., by 1-way ANOVA and Scheffé's multiple comparison test.

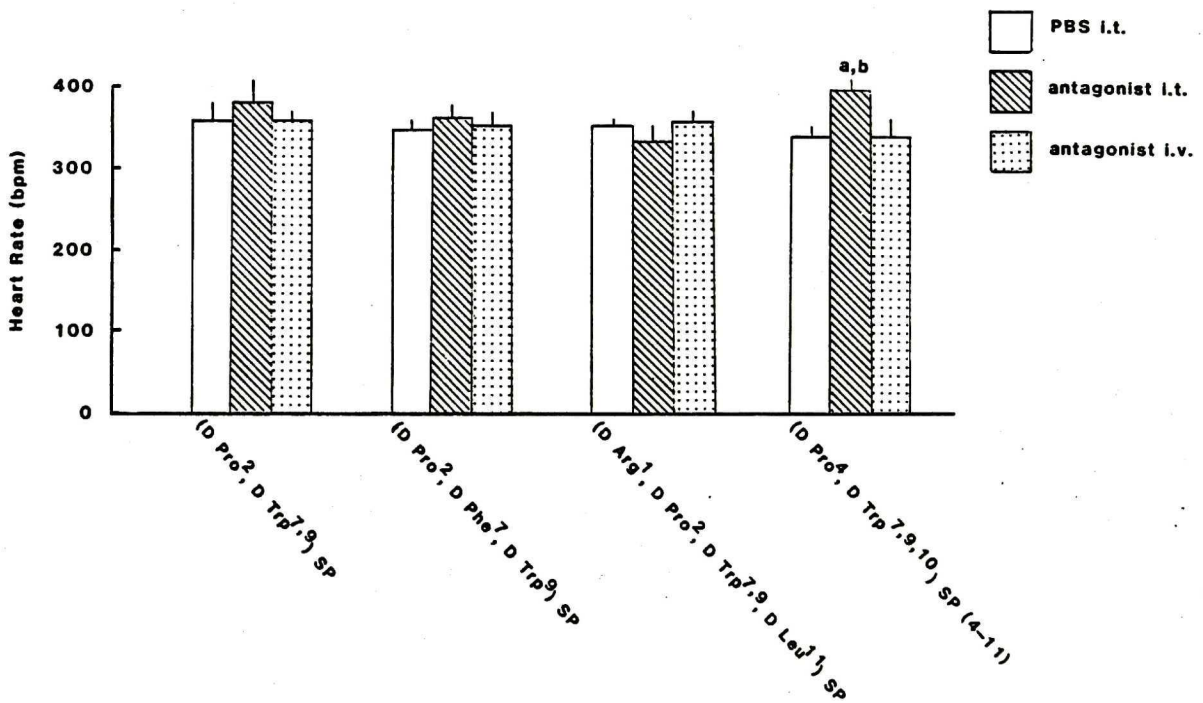

PBS i.t.
antagonist i.t.
antagonist i.v.
Heart Rate (bpm)
400
300
200
100
0
a,b
(D Pro2, D Trp7,9) SP
(D Pro2, D Phe7, D Trp9) SP
(D Arg1, D Pro2, D Trp7,9, D Leu11) SP
(D Pro4, D Trp 7,9,10) SP (4–11)

Figure 26. Increases in mean arterial pressure (MAP) produced by bicuculline methiodide (BMI; 0.59 nmol) applied to the intermediate area of the VSMO, 20 minutes after i.t. injection of SP antagonists (50 mg; cross-hatched bars), i.t. phosphate buffered saline injection (open bars), or i.v. injection of SP antagonists (50 mg; solid bars). I = D-Pro^2, D-Trp^{7,9}-SP. II = D-Pro^2, D-Phe^7, D-Trp^9-SP. III = D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}-SP. IV = D-Pro^4, D-T rp^{7,9,10}-SP(4-11). N = 5-6 rats in each group. | = S.E.M. * = $p<0.05$ comparing responses after PBS i.t. and SP antagonist i.t., † = $p<0.05$ comparing responses after SP antagonist i.t. and SP antagonist i.v., by 1-way ANOVA and Scheffé's multiple comparison test.

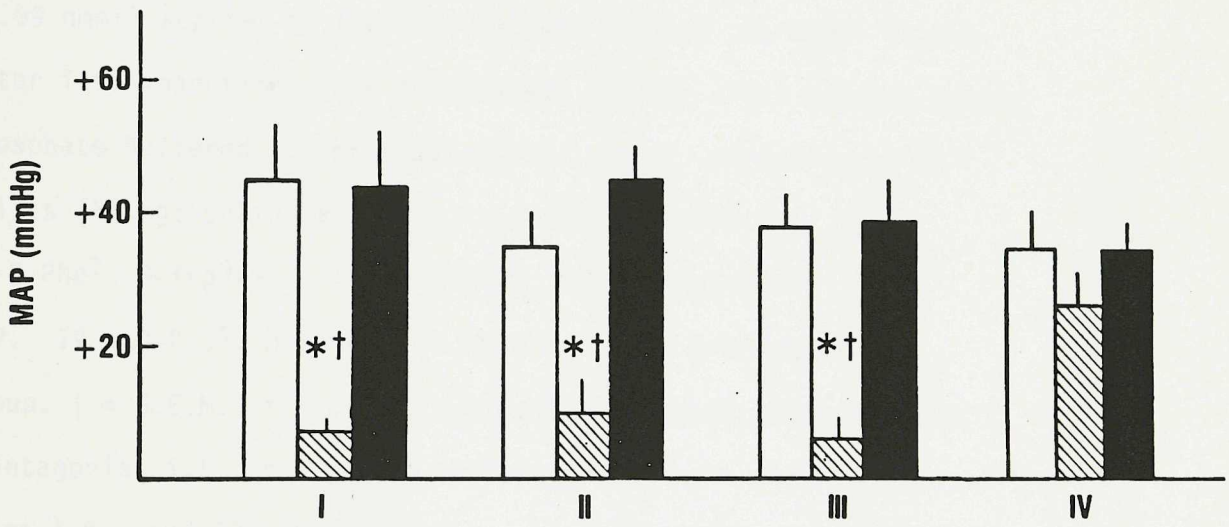

MAP (mmHg)
+60
+40
+20
*†
*†
*†
I
II
III
IV

Figure 27. Increases in heart rate produced by bicuculline methiodide (BMI; 0.59 nmol) applied to the intermediate area of the VSMO, 20 minutes after i.t. injection of SP antagonists (50 mg; cross-hatched bars), i.t. phosphate buffered saline (open bars), or i.v. injection of SP antagonists (50 mg; solid bars). I = D-Pro^{2}, D-Trp^{7,9}-SP. II = D-Pro^{2}, D-Phe^{7}, D-Trp^{9}-SP. III = D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}-SP. IV = D-Pro^{4}, D-Trp^{7,9,10}-SP(4-11). N = 5-6 rats in each group. | = S.E.M. * = $p<0.05$ comparing responses after PBS i.t. and SP antagonist i.t., † = $p<0.05$ comparing responses after SP antagonist i.t. and SP antagonist i.v., by 1-way ANOVA and Scheffé's multiple comparison test.

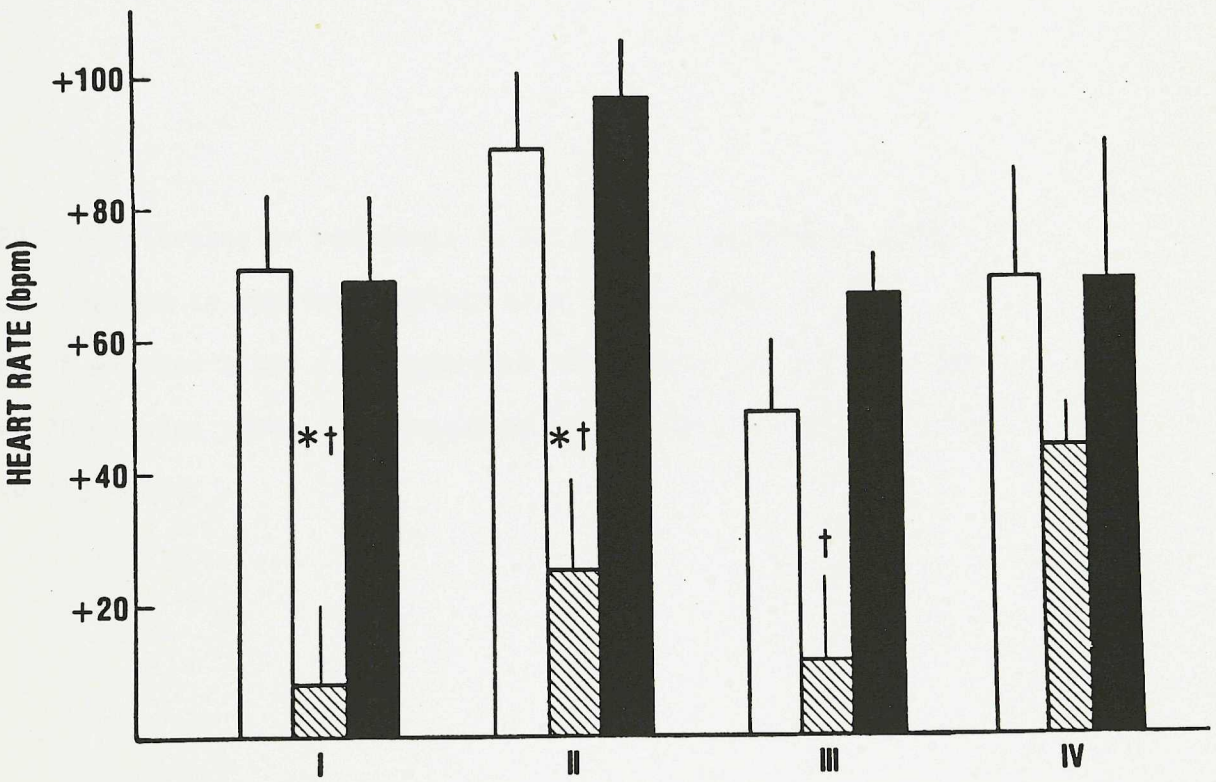

HEART RATE (bpm)
+100
+80
+60
+40
+20
*†
*†
†
I
II
III
IV

Figure 28. Cardiovascular responses to bicuculline methiodide (BMI; 0.59 nmol) applied to the intermediate area of the VSMO. A: BMI responses 20 minutes after i.t. injection of vehicle (15 µl). B: BMI responses 20 minutes after i.t. injection of [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}]-SP (33 nmol).

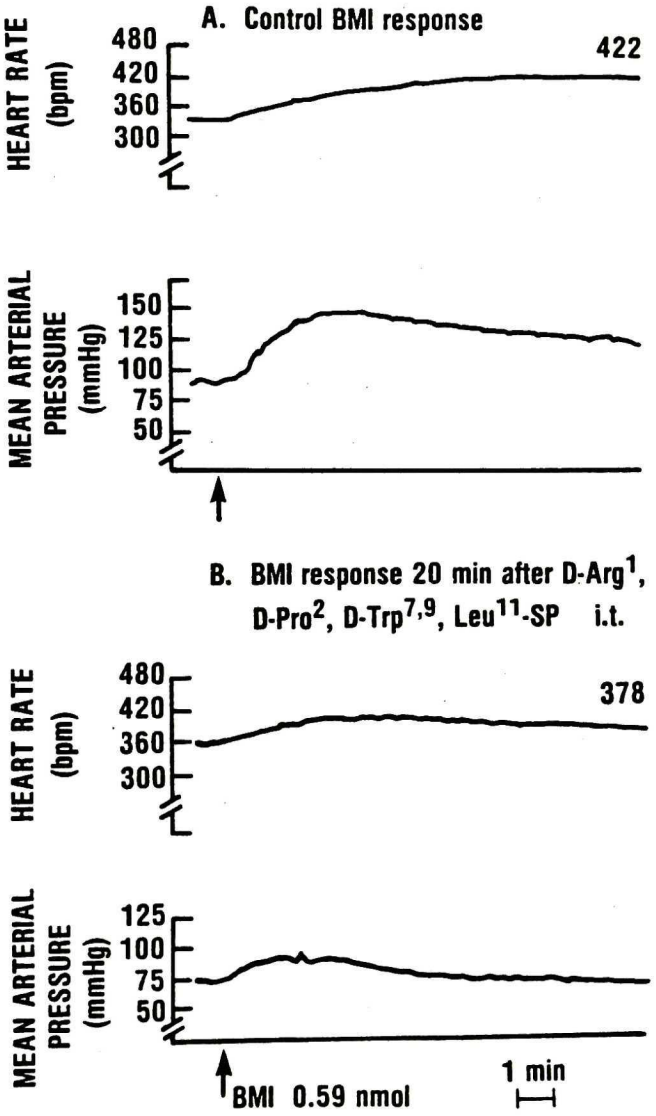

A. Control BMI response
HEART RATE (bpm)
480
420
360
300
422
MEAN ARTERIAL PRESSURE (mmHg)
150
125
100
75
50
B. BMI response 20 min after D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]-SP i.t.
HEART RATE (bpm)
480
420
360
300
378
MEAN ARTERIAL PRESSURE (mmHg)
125
100
75
50
BMI 0.59 nmol
1 min

Figure 29. Changes in mean arterial pressure (MAP) produced by bicuculline methiodide (BMI; 0.59 nmol) applied to the intermediate area of the VSMO, 20 minutes, 1 hour, and 2 hours after [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}]-SP (3.3 nmol i.t.). Baseline MAP values are shown at the bottom of the bars. ( ) = number of rats. | = S.E.M. * = $p<0.05$ compared to baseline MAP prior to BMI application, as measured by 1-way ANOVA and Scheffé's multiple comparison test.

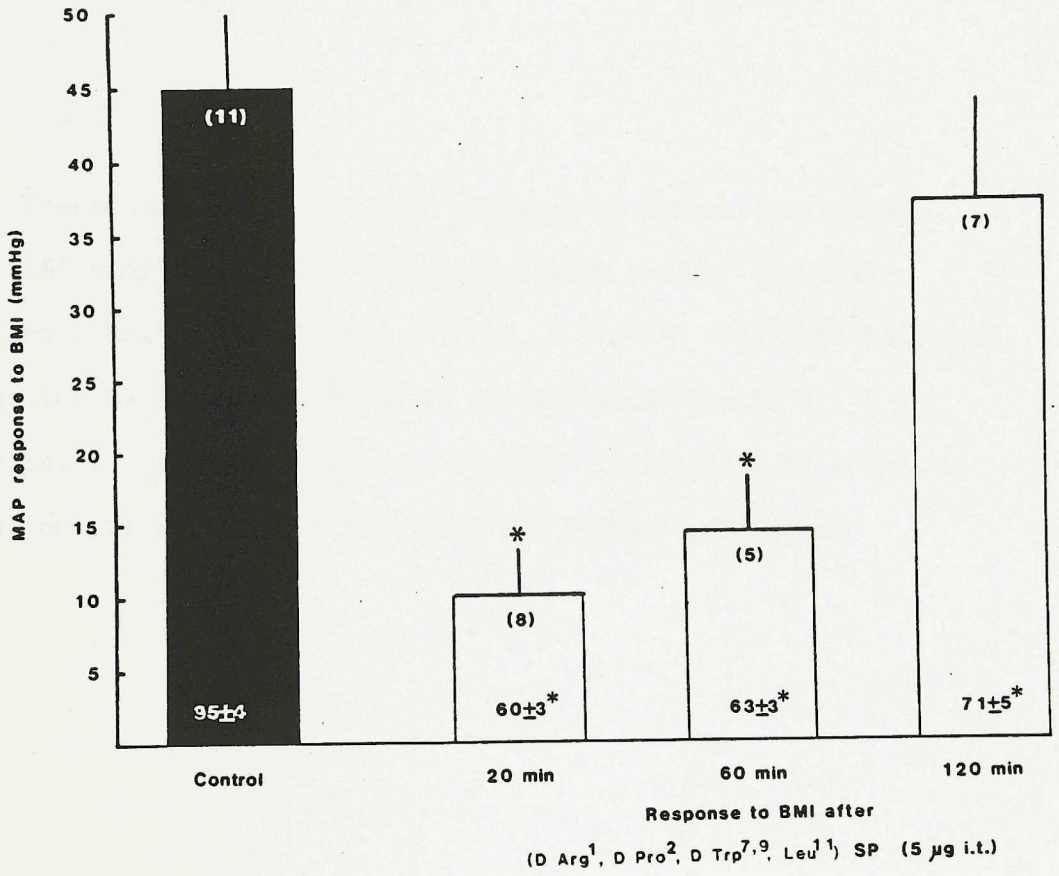
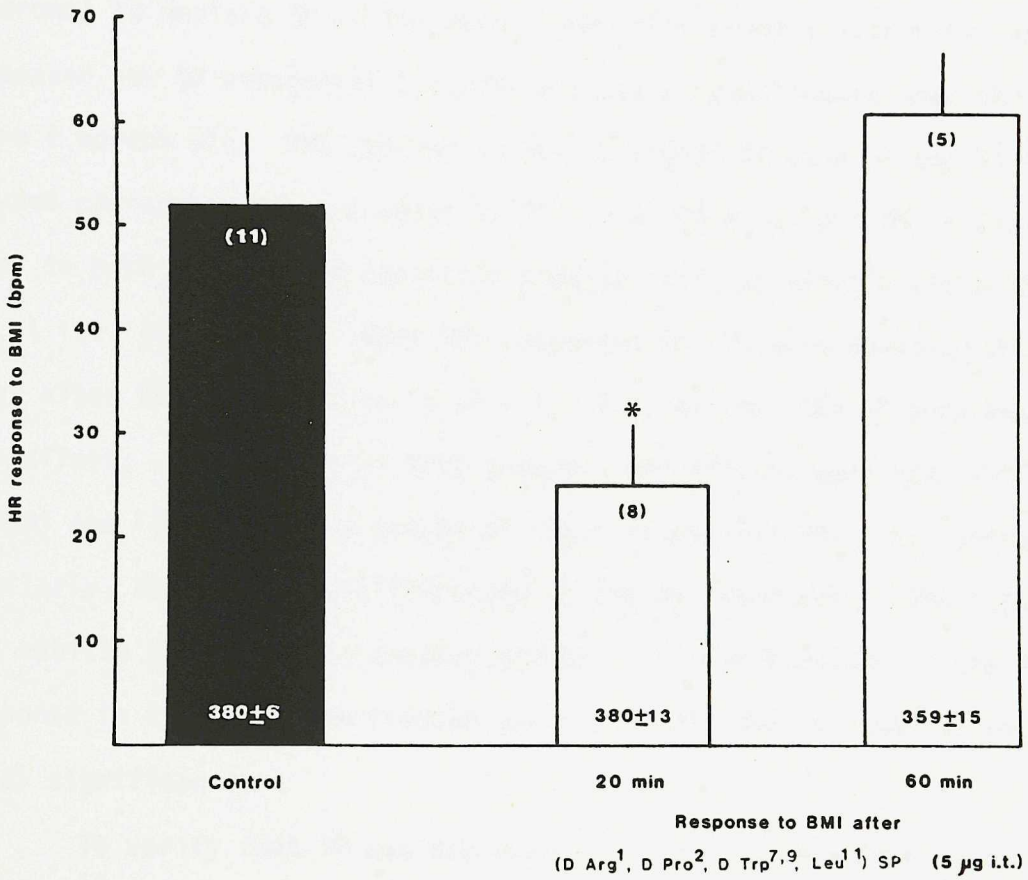

MAP response to BMI (mmHg)
50
45
40
35
30
25
20
15
10
5
(11)
95±4
(8)
60±3*
(5)
63±3*
(7)
71±5*
*
*
Control
20 min
60 min
120 min
Response to BMI after
(D Arg1, D Pro2, D Trp7,9, Leu11) SP (5 µg i.t.)

Figure 30. Changes in heart rate (HR) produced by bicuculline methiodide (BMI; 0.59 nmol) applied to the intermediate area of the VSMO, 20 minutes and 1 hour after [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}]-SP (3.3 nmol i.t.). Baseline HR values are shown at the bottom of the bars. ( ) = number of rats. | = S.E.M. * = $p<0.05$ compared to baseline HR prior to BMI application, by 1-way ANOVA and Scheffé's multiple comparison test.


70
60
50
40
30
20
10
HR response to BMI (bpm)
(11)
380±6
*
(8)
380±13
(5)
359±15
Control
20 min
60 min
Response to BMI after
(D Arg1, D Pro2, D Trp7,9, Leu11) SP (5 µg i.t.)

cord SP was excitatory to the cardiovascular system presumably in the IML, i.t. administration of drugs does not provide site selectivity in the spinal cord. Therefore I also examined the possibility that SP contained in primary afferent systems which terminate in the dorsal horns might also be contributing to these effects. I treated 2 day old rats with capsaicin s.c. (a neurotoxin specific for primary afferent neurons) to deplete SP in the dorsal horn from primary afferents, and repeated the SP antagonist i.t./BMI activation experiments when the rats were 2 months old. BMI applied to the intermediate area of the VSMO caused characteristic increases in MAP (+ 41-63 mmHg) and HR (+ 73-83 bpm) in both vehicle and capsaicin treated rats, 20 minutes after PBS 15 µl i.t. (Figure 31). When MAP responses to BMI were measured 20 minutes after SP antagonist in 15 µl i.t. (i.e. at the time of peak depressor effect; - 18-22 mmHg in both groups), MAP effects were 29% (vehicle group) and 17% (capsaicin group) of their respective PBS i.t. controls. Similarly, there were no differences in the HR responses to BMI between the vehicle and capsaicin treated groups, although blockade of the HR response in the capsaicin treated group did not reach a level of statistical significance.

To verify that SP was depleted from primary afferents by neonatal capsaicin treatment, SP content was measured in the dorsal horn as well as the ventral horn and IML of the thoracic spinal cord. Figure 32 shows that dorsal horn SP was reduced 47%, consistent with previous studies by Helke et al. [1981], Nagy et al. [1980], and Gamse et al. [1980]. There were no differences in SP content in the IML and ventral horn between the two groups.

Figure 31. Bicuculline methiodide-induced (BMI at the intermediate area of the VSMO; 0.59 nmol) pressor (A) and tachycardic (B) responses in vehicle- or capsaicin-treated rats, 20 minutes after phosphate buffered saline i.t. (15 ml; cross-hatched bars), SP antagonist i.t. (3.3 nmol; solid bars), or SP antagonist i.v.(3.3 nmol; dotted bars). N = 5-6 rats in each group. | = S.E.M. * = $p<0.05$ compared to PBS i.t., † = $p<0.05$ compared to SP antagonist i.v., by 2-way ANOVA and Duncan's multiple range test.

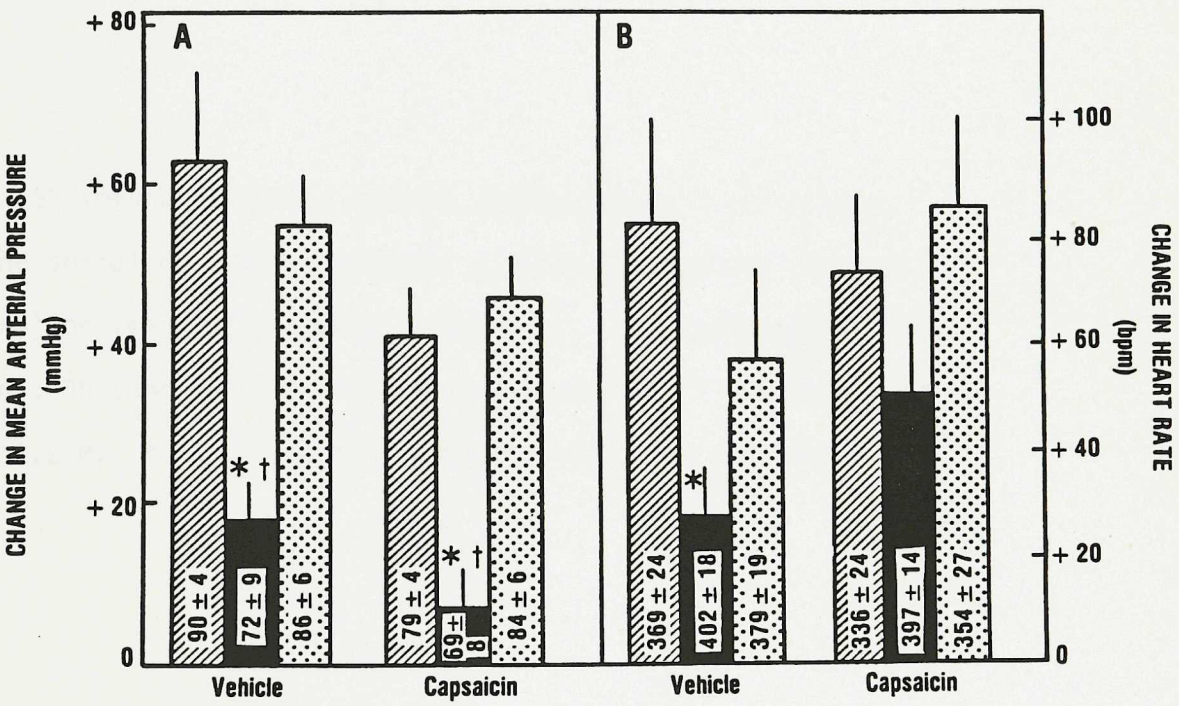
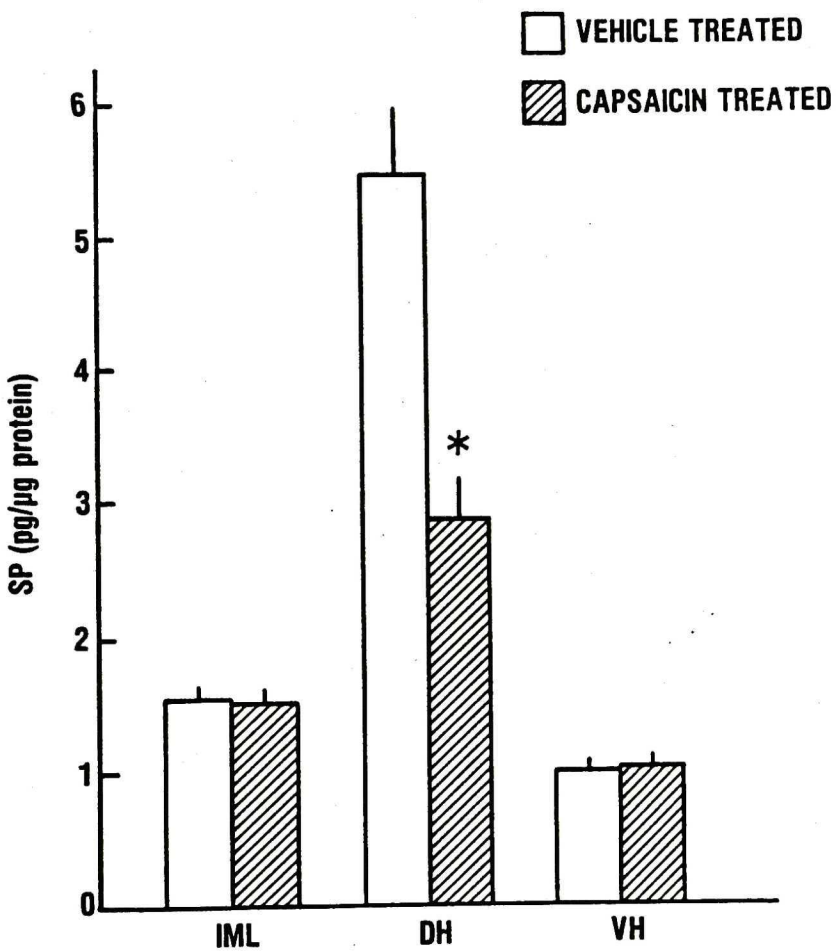

A
B
+ 80
+ 60
+ 40
+ 20
0
+ 100
+ 80
+ 60
+ 40
+ 20
0
CHANGE IN MEAN ARTERIAL PRESSURE (mmHg)
CHANGE IN HEART RATE (bpm)
90 ± 4
72 ± 9
86 ± 6
79 ± 4
69 ± 8
84 ± 6
369 ± 24
402 ± 18
379 ± 19
336 ± 24
397 ± 14
354 ± 27
*
†
Vehicle
Capsaicin
Vehicle
Capsaicin

Figure 32. SP-immunoreactivity content (pg/μg protein) in discrete areas of the spinal cord after neonatal capsaicin treatment (cross-hatched bars) or vehicle treatment (open bars). IML (intermediolateral cell column), DH (dorsal horn), VH (ventral horn). N = 6-9 rats in each group. | = S.E.M. * = $p<0.05$, by Student's *t*-test for unpaired data.


VEHICLE TREATED
CAPSAICIN TREATED
SP (pg/µg protein)
6
5
4
3
2
1
0
*
IML
DH
VH

The results of these SP antagonist studies are consistent with the idea that: 1) SP is a transmitter in an excitatory spinal pathway to the cardiovascular system. This reasoning is based on the fact that i.t. injection of SP antagonists caused a decrease in baseline MAP (Figure 24). 2) The SP-containing pathway mediates its cardiovascular effects via sympathetic outflow from the spinal cord, because a) the cardiovascular effects produced by BMI are mediated by the sympathetic nervous system (Figures 15,16) and SP antagonists blocked these effects, and b) a dorsal horn site of action in the spinal cord was apparently not involved (Figures 31,32). 3) The SP-containing pathway is tonically inhibited by GABA at the VSMO, because i.t. injection of SP antagonists caused a marked reduction in the cardiovascular excitatory responses to disinhibition (of GABA with BMI) at the VSMO (Figures 26-28).

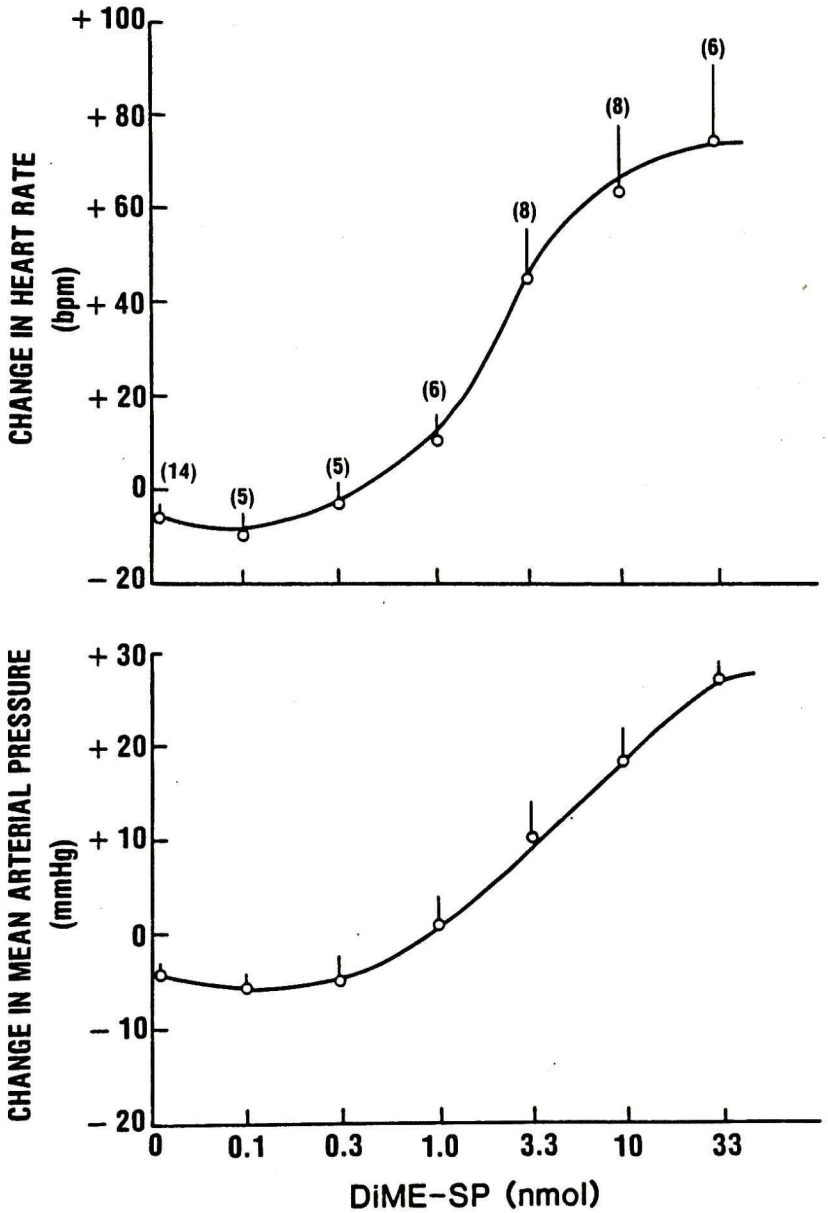
6. Dose-response relationships of SP

I attempted to assess the cardiovascular effects of SP in doses from 6 fmol to 60 nmol i.t. Intrathecal doses high enough to elicit cardiovascular responses (greater than 6 pmol) were indistinguishable from those produced by equal doses given i.v. Immediately after i.t. or i.v. injection, there were dose-dependent depressor responses that returned to baseline values in 5 minutes. There were also tachycardic responses by both routes of administration, but these were dose-related only when SP was given i.v. These results suggested that SP at doses greater than 6 pmol was leaking into the periphery, and SP at doses 6 pmol or less may have been enzymatically inactivated before reaching their presumed target site in the IML.

7. Dose-response relationships of DiME-SP

Because SP i.t. proved to be an ineffective tool to verify the presumed excitatory effects of SP (based on the SP antagonist experiments) on the cardiovascular system, I used the stable active analogue of SP, DiME-SP [Sandberg et al., 1981; Eison et al., 1982a,b], to study receptor interactions in the spinal cord. Evaluation of DiME-SP receptor interactions in rat spinal cord membranes indicated dose-dependent inhibition of saturable, high affinity binding to SP receptors by [^{125}I]-Bolton-Hunter labeled SP [Keeler et al., 1984b]. Intrathecal injection of DiME-SP (0.1-33 nmol) resulted in dose-related pressor and tachycardic responses (Figure 33). Each rat received 1-3 randomized doses. The small depressor and bradycardic responses at lower doses (0.1 and 0.33 nmol) were not signifcantly different from those produced by the PBS vehicle. Typically, an initial depressor response (about 20 mmHg at all doses) in the first 2-3 minutes was followed by a longer lasting pressor response that peaked at about 7 minutes and lasted 30-40 minutes. The tachycardic responses peaked at about 16 minutes with the 3.3 nmol dose lasting up to two hours and higher doses lasting longer than the limits of the observation period (greater than 3 hours). When given intravenously, these same doses of DiME-SP did not cause significant changes in MAP or HR (in the first 2-3 minutes after injection, DiME-SP 10 and 33 nmol evoked depressor responses, - 9-34 mmHg, similar to the effects of SP 6 pmol-2 nmol i.v.). DiME-SP caused tachyphylaxis to repeated i.t. administration. Rats that received a single 33 nmol dose responded with larger increases in MAP and HR (Figure 36) than rats that received as many as 3 randomized doses in these dose-response experiments.

Figure 33. Dose-response relationships (heart rate changes, top; mean arterial pressure changes, bottom) to intrathecal injection of DiME-SP. ( ) = number of rats. | = S.E.M.


+ 100
+ 80
+ 60
+ 40
+ 20
0
– 20
CHANGE IN HEART RATE
(bpm)
(14)
(5)
(5)
(6)
(8)
(8)
(6)
+ 30
+ 20
+ 10
0
– 10
– 20
CHANGE IN MEAN ARTERIAL PRESSURE
(mmHg)
0
0.1
0.3
1.0
3.3
10
33
DiME-SP (nmol)

8. Reversal of substance P antagonist with DiME-SP

To further verify that SP antagonists were working through SP receptors to produce their cardiovascular effects, I attempted to reverse the blockade caused by SP antagonist III (3.3 nmol i.t.). In control animals, the baseline MAP was decreased about 30 mmHg by the SP antagonist but the depressor response was prevented by i.t. injection of DiME-SP (33 nmol; Figure 34). In addition, the BMI-induced pressor response (+45 mmHg) was blocked at 20 minutes and 60 minutes after the i.t. injection of SP antagonist but returned to control levels at 120 minutes (Figure 29). The blockade of the BMI-induced pressor response by the SP antagonist could also be prevented by i.t. injection of DiME-SP (Figure 34). This effect was seen 20 minutes following the administration of SP antagonist III i.t., the time point shown to be the peak effect of the SP antagonist.

9. Interactions of DiME-SP with the sympathetic nervous system

To determine the role of the sympathetic nervous sytem in the cardiovascular actions of DiME-SP the MAP and HR responses to DiME-SP (33 nmol i.t.) were assessed in the absence or presence of ganglionic and β-adrenoreceptor blockers given i.v. The experimental protocol is schematically shown in Figure 35. The pressor response to DiME-SP was blocked by prior (3-5 minutes) administration of pentolinium 10 mg/kg i.v. (Figure 36). There was no change in the baseline HR after pentolinium. The tachycardic response to DiME-SP was markedly reduced by prior (3-5 minutes) injection of propranolol 1 mg/kg i.v. (Figure 36) as well as by pentolinium 10 mg/kg i.v. (19% of the DiME-SP response in the PBS group, data not shown). Plasma catecholamines assayed from blood drawn 7 minutes after DiME-SP i.t. (corresponding to the peak MAP

Figure 34. Bicuculline methiodide (0.59 nmol)-induced (topically applied to the intermediate area of the VSMO) pressor responses before, 20 minutes and 60 minutes after i.t. phosphate buffered saline (15ml; hatched bars PBS (hatched bars), [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}]-SP (3.3 nmol; solid bars), or DiME-SP (33 nmol) and [D-Arg^{1}, D-Pro ^{2}, D-Trp^{7,9}, Leu^{11}]-SP (3.3 nmol; dotted bars). Numbers at the bottom of each histogram are baseline mean arterial pressure values (mmHg) prior to topical application of BMI. ( ) = number of rats. | = S.E.M. * = $\underline{p} < 0.05$ compared to control BMI response by 1-way ANOVA followed by Duncan's multiple range test.

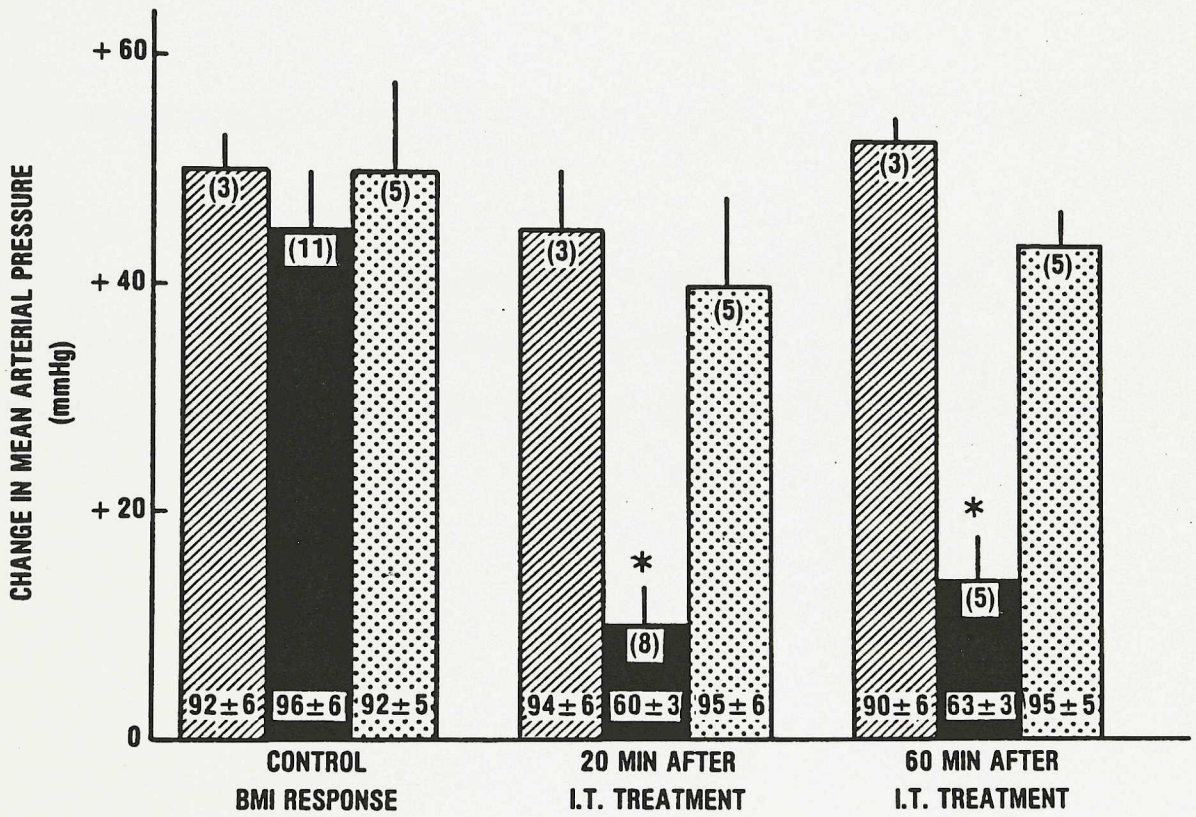

CHANGE IN MEAN ARTERIAL PRESSURE (mmHg)
+ 60
+ 40
+ 20
0
(3)
(11)
(5)
(3)
(5)
(3)
(5)
(8)
(5)
*
*
92±6
96±6
92±5
94±6
60±3
95±6
90±6
63±3
95±5
CONTROL BMI RESPONSE
20 MIN AFTER I.T. TREATMENT
60 MIN AFTER I.T. TREATMENT

Figure 35. Protocol for DiME-SP/catecholamine experiments. Blood was drawn 7 minutes after phosphate buffered saline (PBS) i.t., 7 minutes after DiME-SP i.t. and again 9 minutes later (16 minutes after DiME-SP i.t.).

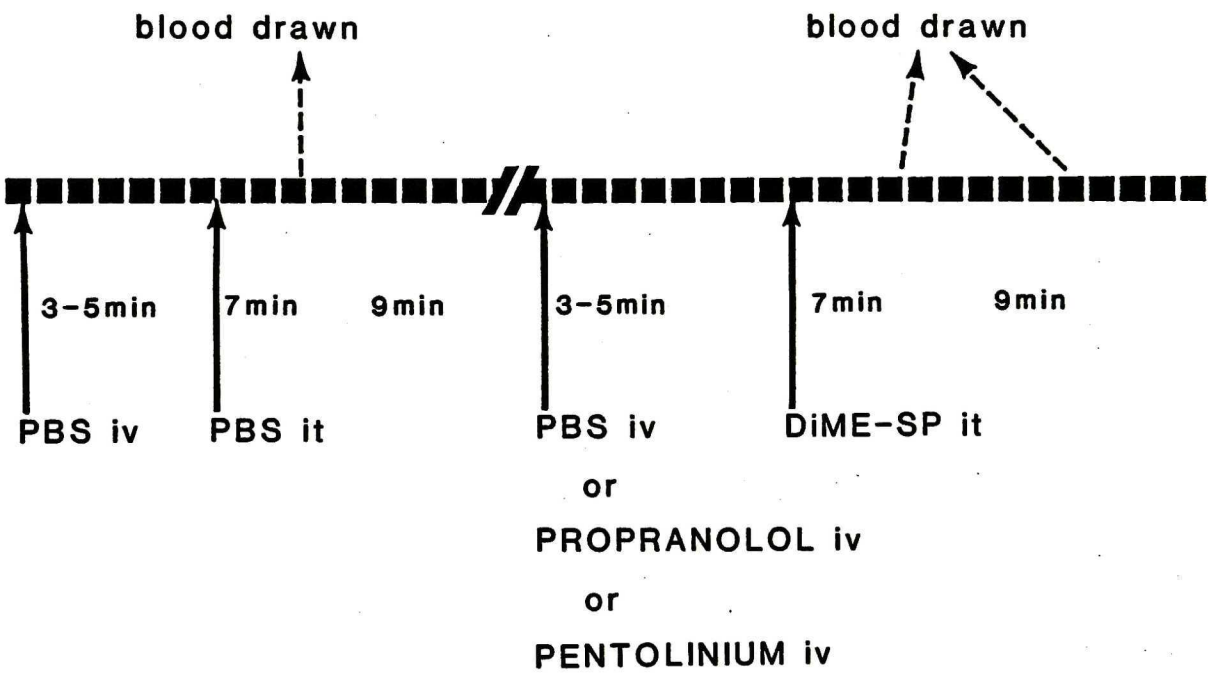
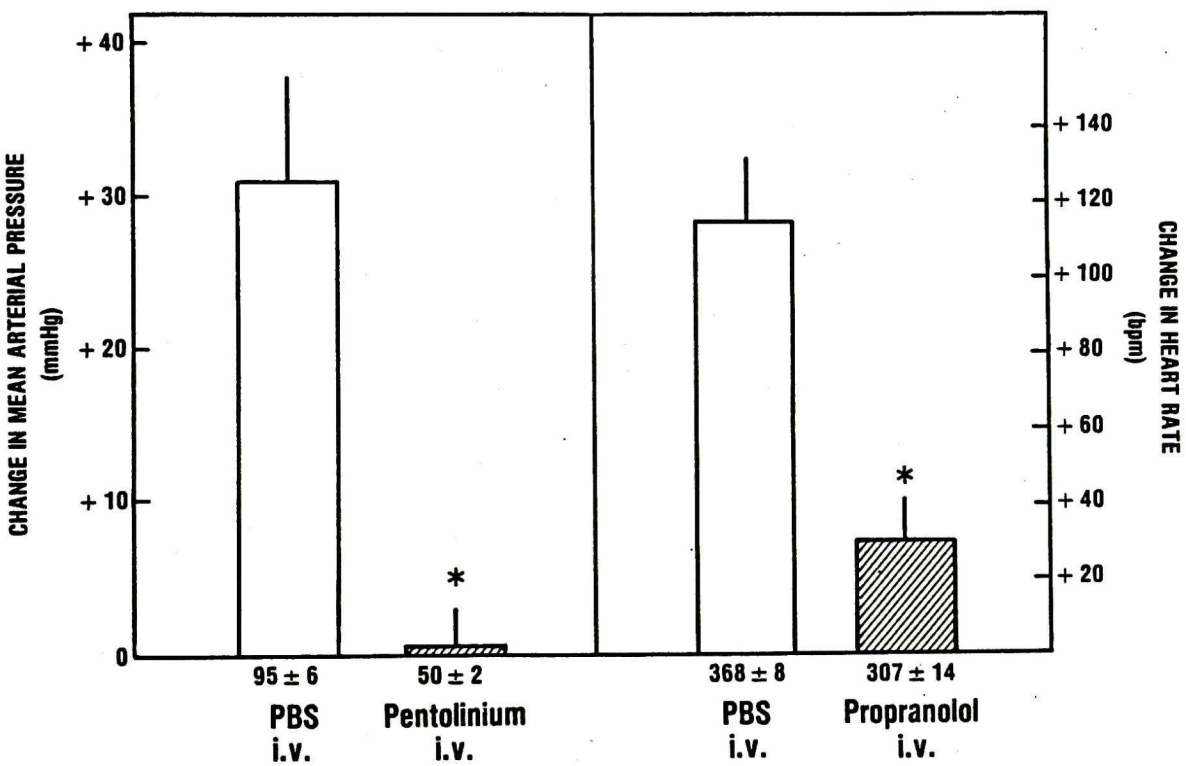

blood drawn
blood drawn
3-5min
7min
9min
3-5min
7min
9min
PBS iv
PBS it
PBS iv
or
PROPRANOLOL iv
or
PENTOLINIUM iv
DiME-SP it

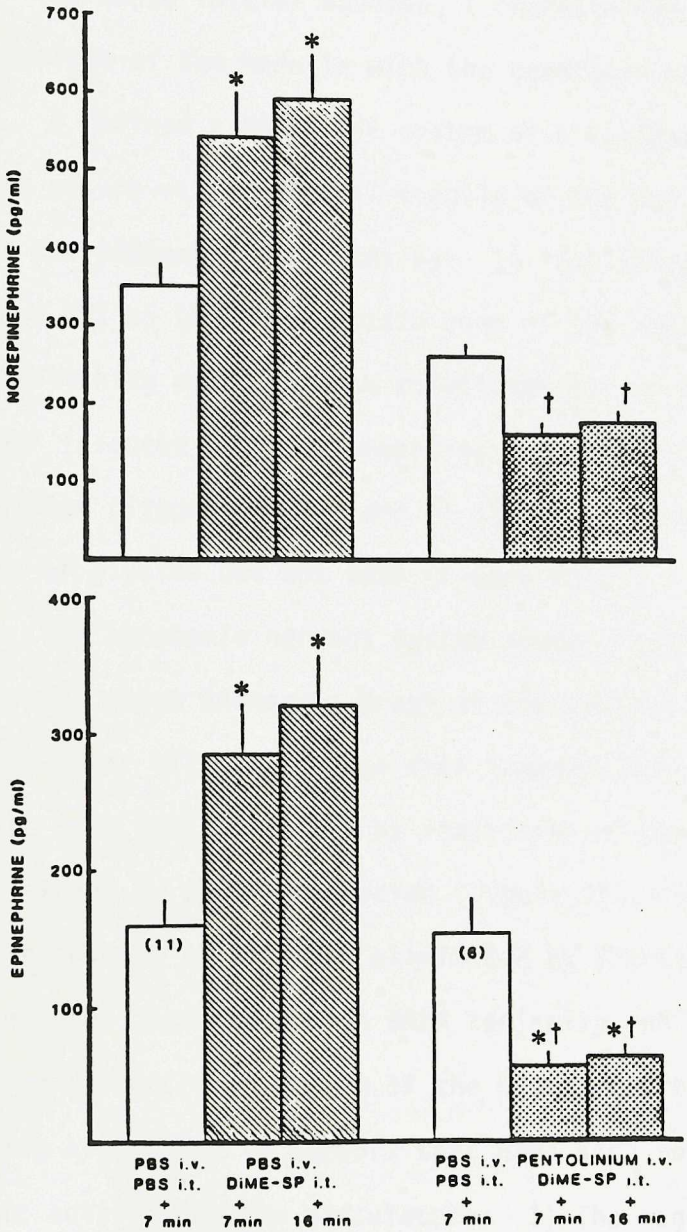
Figure 36. Cardiovascular effects of DiME-SP (33 nmol i.t.) preceded 3-5 minutes by phosphate buffered saline (PBS; open bars), pentolinium or propranolol i.v. (hatched bars). N = 6 rats in each group. Baseline mean arterial pressure (mmHg) heart rate (bpm) are shown at the bottom of the histograms. | = S.E.M. * = $\underline{p} < 0.05$ by Student's $\underline{t}$-test for unpaired data.


CHANGE IN MEAN ARTERIAL PRESSURE (mmHg)
+ 40
+ 30
+ 20
+ 10
0
CHANGE IN HEART RATE (bpm)
+ 140
+ 120
+ 100
+ 80
+ 60
+ 40
+ 20
*
*
95 ± 6
50 ± 2
368 ± 8
307 ± 14
PBS i.v.
Pentolinium i.v.
PBS i.v.
Propranolol i.v.

effect) were elevated to 163% (epinephrine) and 154% (norepinephrine) of values obtained after PBS i.t. Nine minutes later (corresponding to the peak HR effect) catecholamine levels remained elevated. Prior injection of pentolinium i.v. decreased basal unstimulated (i.e. from blood drawn 7-10 minutes after PBS i.t.) levels of catecholamines and reduced the DiME-SP stimulated levels to 20% (epinephrine) and 40% (norepinephrine) of their vehicle i.v. controls (Figure 37).

The results of these DiME-SP studies further enhanced the idea that: 1) SP is a transmitter in an excitatory spinal pathway to the cardiovascular system, because the SP agonist DiME-SP caused increases in MAP and heart rate, which is consistent with the opposite effects produced by the SP antagonists. The dose-related increases in MAP and HR suggest that these actions were mediated via SP receptors (Figure 33). 2) The SP-containing pathway mediates its cardiovascular effects via sympathetic outflow from the spinal cord, because a) DiME-SP countered the blockade (produced by a SP antagonist) of the cardiovascular effects of BMI which are mediated by the sympathetic nervous system (Figure 34); b) the excitatory cardiovascular effects produced by i.t. injection of DiME-SP are accompanied by increases in plasma catecholamines (Figure 37), and both responses can be blocked by peripherally administered sympathetic blockers (Figures 36,37). 3) The SP-containing pathway is tonically inhibited by GABA at the VSMO, because i.t. injection of DiME-SP blocked the SP antagonist mediated inhibitory effect on BMI-induced excitatory responses (Figure 34).

Figure 37. Plasma epinephrine (bottom) and norepinephrine (top) responses to DiME-SP (33 nmol i.t.). DiME-SP was preceded 3-5 minutes by phosphate buffered saline (PBS) vehicle i.v. (hatched bars) or pentolinium 10 mg/kg i.v. (cross-hatched bars) given in equal volumes. Blood was drawn 7 minutes (corresponding to peak blood pressure effects) and 16 minutes (corresponding to peak heart rate effects) after i.t. drug administration. ( ) = number of rats. | = S.E.M. * = different from control value (PBS i.v./PBS i.t. + 7 minutes, open bars). † = different from PBS i.v./DiME-SP i.t. group at the respective time point. $\underline{p} < 0.05$ by 2-way ANOVA with Duncan's multiple range test.


NOREPINEPHRINE (pg/ml)
700
600
500
400
300
200
100
EPINEPHRINE (pg/ml)
400
300
200
100
(11)
(6)
PBS i.v.
PBS i.t.
+
7 min
PBS i.v.
DiME-SP i.t.
+
7min
+
16 min
PBS i.v.
PBS i.t.
+
7 min
PENTOLINIUM i.v.
DiME-SP i.t.
+
7 min
+
16 min

DISCUSSION

The Ventral Surface of the Medulla in the Rat: Pharmacologic and Autoradiographic Localization of GABA-induced Cardiovascular Effects

In these initial studies, I characterized interactions of the ventral surface of the medulla with the cardiovascular system in a rat model. I defined a GABAergic system of a vasodepressor and negative chronotropic nature at the ventral medulla of the rat. I verified the specificity of the GABAergic responses by: 1) localizing the maximum responses of GABA and BMI to the intermediate zone of the ventral surface (Figure 14); 2) establishing dose-response relationships for the effects of GABA and muscimol (Figures 5,6); 3) reversing the effects of GABA and muscimol with bicuculline (Figures 9-11); and 4) showing that strychnine reversed the effects of glycine but not GABA (Figure 12).

The autonomic nervous system appears to be the final common pathway through which GABAergic drugs at the ventral medulla mediate their cardiovascular effects. These data suggest that GABA produces a decrease in heart rate (HR) primarily by inhibition of sympathetic outflow and to a lesser extent by vagal activation (Figure 7). In addition, that the tachycardic responses to BMI were eliminated by intravenous propranolol (Figure 15) indicates that endogenous GABA tonically inhibits sympathetic activity governing chronotropic action of the heart. Furthermore, based on the following arguments, it appears that GABA also tonically reduces the sympathetic activity to the vasculature: 1) The magnitude of the reductions in mean arterial pressure (MAP) due to intravenous phentolamine and topically applied GABA were similar (Figure 8). 2) GABA did not further reduce the MAP in the presence of peripherally administered phentolamine (Figure

8). 3) After phentolamine administration, the additional drop in blood pressure produced by a direct acting vasodilator (hydralazine) indicated that the blood vessels were still capable of further dilatation. 4) Prevention of the pressor response in BMI by blockade of another site in the peripheral sympathetic pathway (with pentolinium; Figure 16) further verified the GABAergic inhibitory influence over sympathetic outflow to the vasculature.

To learn if the pressor responses to BMI might be mediated in part by vasoactive hormones, I also assessed its interactions with the renin-angiotensin system and its ability to stimulate vasopressin release from the posterior pituitary in four rats. Captopril (2 mg/kg i.v.), an angiotensin converting enzyme inhibitor, decreased the blood pressure (- 24 mmHg) and blocked the pressor (+ 20 mmHg) effects of angiotensin I (200 ng/kg i.v.). Captopril did not block the pressor responses to BMI (0.59 nmol) topically applied to the VSMO, nor the pressor responses to angiotensin II (200 ng/kg i.v.; indicating that the angiotensin II receptors were functional). A vasopressin blocker specific for vascular receptors, [1-(β -Mercapto-β,β-cyclopentamethylene proprionic acid),2-(O-methyl) tyrosine] Arg^8-vasopressin (35 µg i.v.), decreased the blood pressure (- 18 mmHg) and blocked the pressor effects (+ 90 mmHg) to arginine vasopressin (0.5 µg i.v.), but not those produced by BMI (0.59 nmol) topically applied to the VSMO. These results were further indications that the cardiovascular effects of BMI were mediated probably exclusively by the autonomic nervous system.

The data obtained from these studies demonstrate that the rat is a suitable animal model in which to study the functional aspects of the ventral medulla. The responses to GABAergic drugs applied to the ventral

medulla are similar to those obtained in the cat: 1) Bradycardic and hypotensive responses were elicited by topical application of GABA agonists [Guertzenstein, 1973; Wennergren and Oberg, 1980; Feldberg and Rocha e Silva, 1981; Yamada et al., 1982] (Figures 3-11). 2) The most sensitive region in the rat is the intermediate area. This area is equivalent to Schlaefke's area, the area most sensitive to topical application of GABA in the cat [Feldberg, 1976; Yamada et al., 1982] (Figure 4). 3) As in the cat, the effects of GABA were primarily mediated by sympathetic inhibition [Antonaccio and Taylor, 1977] (Figures 7,8). In addition, this was the first demonstration in any species that the cardiovascular effects of GABAergic drugs at the VSMO specifically, were mediated by the sympathetic nervous system. The overshoot of HR and MAP upon reversal of GABA by bicuculline (Figure 9) was a statistically significant change when compared to the magnitiude of decreases elicited by the prior GABA application (the trend did not reach a level of significance for muscimol reversal, Figure 10). This suggested that bicuculline was reversing not only the exogenously applied GABA but perhaps an endogenous GABA component as well. Application of bicuculline alone to the intermediate area substantiated this finding (Figure 13) and suggested that endogenous GABA exerts a tonic inhibitory influence on cardiovascular function.

The tachycardic response to bicuculline is one effect that seems peculiar to the rat. While Yamada et al. [1982] studied the effects of bicuculline on both HR and MAP in cats, they found statistically significant increases only in MAP. The magnitude of the increases I observed in the rat indicates a considerable tonic inhibitory effect of GABA on HR as well.

Because of the extensive vascularity of the VSMO, it has been

suggested that drugs topically applied, and therefore thought to act on neurons near the ventral surface, are perhaps taken up by the vasculature and transported, or they may diffuse along perivascular spaces to other structures [Borison et al., 1980]. However, several lines of evidence suggest that this does not occur. For example, microinjections of GABA into discrete areas more typically noted for their cardiovascular effects, i.e. the nucleus of the solitary tract [Persson, 1981; Bousquet et al., 1982a], nucleus ambiguus [DiMicco et al., 1979; Blessing and Reis, 1983], and the A1 region [Blessing and Reis, 1982, 1983] increased blood pressure, whereas my data show that GABA applied to the intermediate area of the VSMO decreases blood pressure. In addition, microinjections of glutamate into the A5 region also decrease blood pressure [Neil and Loewy, 1982; Stanek et al., 1984]. Therefore, it is likely that the cardiovascular responses I observed were mediated at a site distinct from these more commonly studied areas. In addition, pledget application of [^{3}H]GABA confirmed drug localization to ventral structures (Figures 17,18). Labeling was concentrated in a region that most closely corresponds to the lateral paragigantocellular nucleus (PGCL) [Andrezik et al., 1981a]. Because of the rostrocaudal and lateral spread of radiolabeled drug, there are other possible anatomic substrates for the cardiovascular effects of drugs applied to the VSMO. However, the area of greatest drug sensitivity was localized to the intermediate zones (Figures 4,14), corresponding also to the PGCL. The discrete localization of sensitivity to GABAergic drugs at the VSMO/PGCL in rats has since been confirmed in other laboratories by microinjection [Willette et al., 1983b] or topical application [Benarroch et al., 1984]. The absence of tritium counted in peripheral blood further indicated that GABA's actions were central.

Given the short period of exposure to the [^{3}H]GABA to the VSMO in vivo (2 minutes), the tritium detected in this study by either autoradiography (Figure 18) or scintillation spectrometry (Figure 17) is likely to be primarily [^{3}H]GABA. However, the possibility exists that more readily diffusible tritiated metabolites of GABA are formed and therefore the extent of penetration of GABA into brain parenchyma may be less than 1 mm.

These studies present evidence for cardiovascular modulation at the VSMO in the rat. My findings show a GABAergic inhibitory influence on sympathetic outflow, and this modulation appears to be localized to structures at or near the surface at the intermediate area of the ventral medulla.

Spinal cord substance P mediates the sympathoexcitatory cardiovascular responses evoked by GABA disinhibition at the ventral surface of the medulla.

These studies provide evidence that SP is a functional component of a sympathoexcitatory bulbospinal pathway that is inhibited by GABA at the VSMO. The sympathetic excitation which results from disinhibition by the GABA-receptor antagonist, BMI, is obtunded by SP antagonists injected i.t., but not by i.t. treatment with the 5-HT neurotoxin 5,7-dihydroxytryptamine (5,7-DHT). My data also suggest that while SP mediates excitatory effects on both MAP and HR, these may be intrinsically separate pathways based on differential time courses and responses to SP antagonists. While the precise site of action of SP antagonists in the spinal cord was not elucidated, the dorsal horn does not appear to be a major site for the cardiovascular actions observed. Blockade of BMI-induced (at the VSMO) increases in MAP and HR, by SP receptor antagonism in the spinal cord, was reversible with time. The role of SP in spinal sympathetic pathways that

influence the cardiovascular system was further elucidated in the DiME-SP experiments. I characterized the cardiovascular responses to intrathecal administration of DiME-SP and established their contingency on the peripheral sympathetic nervous system. I provided further evidence that the excitatory cardiovascular effects evoked by stimulation of cell bodies at the VSMO were due largely to SP transmission in the spinal cord.

The results of this second series of experiments were derived largely from observing the cardiovascular effects produced by intrathecal administration of drugs. This technique had been used successfully in rats to evaluate the effects of drugs on neural activity recorded directly from the lumbar sympathetic chain [LoPachin and Rudy, 1981]. Nevetheless, it was necessary to confirm that i.t. pharmacologic manipulation of sympathetic activity could be expressed and monitored in terms of cardiovascular changes. In preliminary experiments, I injected procaine (5%) i.t. in two rats. This decreased MAP and HR to the same degree as GABA applied to the VSMO, or sympathetic blockers injected i.v. Intrathecally administered local anesthetics are known to block sympathetic outflow [see review, Murphy, 1981], and provided a preliminary indication that this could be an effective system for clarifying the role of neurotransmitters in spinal pathways in the cardiovascular system in rats.

The pressor and tachycardic responses to BMI in the presence of i.t. vehicle or i.v. antagonists (Figures 21,23,26,27) were consistent with those shown in previous experiments (Figures 13-16,19), thus i.t. catheterization per se did not alter the responses. In addition, responses to i.t. agonists and antagonists were not caused by a nonspecific volume effect in the spinal cord because equal volumes of i.t. PBS had no effect on MAP and HR, nor were they caused by peripheral leakage because an equal

dose of i.v. agonists and antagonists (not true for the bradycardic effects of cinanserin) had no effect. An equal volume of i.t. injected dye was confined to the spinal subarachnoid space.

The evidence for a VSMO-IML pathway [Loewy and McKellar, 1981] coupled with the excitatory effects of 5-HT on sympathetic preganglionic neurons [deGroat and Ryall, 1967; Coote et al., 1981; McCall, 1983] prompted me to investigate if spinal 5-HT was the mediator of the cardiovascular effects I had seen produced by disinhibition of GABA at the VSMO.

Intrathecal administration of the 5-HT receptor antagonist, cinanserin, decreased MAP and HR which was consistent with a toxically active VSMO-IML pathway (Figure 21). In addition, the BMI-induced (at the VSMO) pressor and tachycardic responses were reduced by 50%, which suggested also that these effects were functionally linked with the VSMO. However, further attempts to verify these results with i.t. treatment with the 5-HT neurotoxin, 5,7-DHT, were contradictory (Figure 23). Depletion (56%) of spinal cord 5-HT (Figure 22) did not decrease baseline MAP and HR, nor modify the normal cardiovascular responses to BMI or GABA applied topically to the VSMO. These results were in agreement with those of Loewy and Sawyer [1982], who reported that a reduced population of serotonergic neurons in the ventral medulla (due to intracisternal 5,7-DHT injections) did not change the pressor or tachycardic response to topical application of kainic acid at VSMO. On the other hand, Howe et al. [1983b] reported as much as 66% decrease in the pressor response to electrical stimulation in rats treated with intraspinal 5,7-DHT. Although these authors injected glutamine and kainic acid into the same sites that were electrically stimulated to verify pressor responses to activation of cell bodies, they did not examine the responses to these excitatory amino acids after 5,7-DHT treatment. It is

possible that the axons of the 5-HT pressor pathway they described, passed through the VSMO and were electrically stimulated, whereas another pressor pathway was chemically stimulated. Alternatively, 1) the 5-HT pressor pathway that originates in the VSMO is not tonically inhibited by GABA, or 2) this is such a powerful pathway, that 90% but not 56% depletion of 5-HT in the thoracic spinal cord was necessary to unmask the contribution of 5-HT in these responses.

Recently, McCall [1984] reinvestigated the origin of this 5-HT pathway. He stimulated pressor sites in areas of the medulla known to contain 5-HT neurons that projected to sympathetic preganglionic neurons. Sympathoexcitatory responses (recorded from the inferior cardiac nerve) were evoked by stimulation of medial (nuclei raphe magnus, obscurus, and pallidus) and lateral (area of VSMO/PGCL) sites, however only medial site-evoked responses were blocked by intravenous 5-HT antagonists (doses previously shown to decrease spontaneous and 5-HT-evoked sympathetic discharges [McCall, 1983]. These results provided more conclusive evidence that the 5-HT sympathoexcitatory pathway does not originate from the 5-HT neurons at the VSMO, and confirm the results of my 5,7-DHT study as well as that of Loewy and Sawyer [1982].

The earlier results from my cinanserin experiments were misleading, however there is a possible explanation for the data presented in Figure 21: The dose necessary to block the cardiovascular effects of BMI applied to the VSMO was 350 µg. Assuming that the drug was distributed throughout the spinal subarachnoid space (as implied by the distribution of dye injected in the same 15 µl volume), the concentration was about 12 mM. Leysen *et al.* [1981] determined the receptor binding profiles of several 5-HT antagonists in rat and guinea pig brain homegenates. The inhibitory

potencies of cinanserin (K_i values) for 5-HT_2 and 5-HT_1 receptors were 41 nM and 3.5 μM, respectively. Therefore, the dose necessary to block the cardiovascular effects of BMI in my experiments was 1-2 orders of magnitude higher than these reported concentrations. Although cinanserin undoubtedly had better access to receptor sites in Leysen's study and rat spinal cord homogenates were not used in these studies, it is conceivable that the results from my experiments were due to interaction with non-5-HT receptors or even a nonspecific "local anesthetic" effect. Indeed, Leysen reported that cinanserin also interacted with other receptors (K_i values for histamine, α_1, and dopamine receptors were 1.2, 1.2, 1.6 μM, respectively) whose inhibitory potencies were well below the dose I found to be effective. In addition, results from Leysen's study indicated that methysergide was 3.4 times more potent as a 5-HT_1 receptor antagonist, and 35 times more potent as a 5-HT_2 receptor antagonist than cinanserin. If cinanserin was acting through 5-HT receptors in my experiments, theoretically I should have been able to reproduce my cinanserin data with methysergide in the 170 to 340 nmol dose range. Methysergide has been used successfully when injected i.t. in rats to characterize spinal 5-HT receptors involved in nociception. Methysergide dose-dependently antagonized the increased tail flick latency produced by i.t. injected 5-HT and the ID_{50} was calculated to be 17.5 nmol [Schmauss et al., 1983]. This dose was 19 times smaller than the highest dose of methysergide that I injected and which did not antagonize the cardiovascular effects to BMI at the VSMO. One could argue that the inferred site of action of methysergide in the dorsal horn in Schmauss' study was closer to the spinal subarachnoid space than the IML in my experiments; however, methysergide's lipophilicity [Merck Index, 1976] is suggestive of potential

to penetrate the white matter of the spinal cord to reach the IML.

Results from the SP studies on the other hand, were consistent. The baseline MAP was significantly reduced by i.t. SP antagonists I, II and III, to 58-65 mmHg (Figure 24). These data are consistent with the idea of a tonically active pathway and with the results of other studies that implicate spinal SP as excitatory to sympathetic outflow in rats [Loewy and Sawyer, 1982; Gilbey et al., 1983; Yashpal et al., 1983; Takano et al., 1984a,b].

The results from this study also imply that SP neurons in the spinal cord are subject to GABA inhibition at the VSMO. Blockade of GABA receptors at the VSMO with bicuculline results in sympathetic excitation to the cardiovascular system (Figures 15,16), an effect which was significantly attenuated after i.t. administration of SP antagonists I, II and III (Figures 26,27). Coupled with evidence for SP binding sites in the IML [Charlton and Helke, 1984b; Maurin et al., 1984] and knowledge of a descending ventral medullary - IML SP projection [Helke et al., 1982], these data support the concept that ventral medullary application of bicuculline activates excitatory bulbospinal SP neurons which transmit the response to the sympathetic nervous system via the IML. Theoretically, dorsal horn SP derived from nerve terminals to the central branches of primary afferents [Takahashi and Otsuka, 1975; Hökfelt et al., 1975b; Barber et al., 1979] could also have been a site of the SP antagonist actions I observed. These SP pathways are thought to be concerned with sensory modalities [Yaksh et al., 1979; Piercey et al., 1981a,b] and could be involved with somatosympathetic [reviews by Koizumi and Brooks, 1972; Sato and Schmidt, 1973] and viscerosympathetic [Franz et al., 1966] spinal cardiovascular reflexes. Therefore, SP blockade in the dorsal horn

could theoretically interfere with either of these processes and alter cardiovascular function. In my capsaicin experiments, neonatal capsaicin treatment decreased SP-immunoreactivity content in the dorsal horn by 47% (consistent with almost total SP depletion in dorsal roots [Nagy et al., 1981] (Figure 32), but did not alter the cardiovascular responses to SP antagonist III i.t., BMI applied to the VSMO, or SP antagonist III's blockade of BMI (Figure 31). These results indicate that the SP antagonist probably did not have its major action by blocking SP from primary sensory neurons. Finally, this is the first report of capsaicin effects on IML SP-immunoreactivity content. That SP-I content in the IML was not changed by neonatal capsaicin treatment (Figure 32) further supports the selectivity of capsaicin for primary afferent neurons, since central terminals of primary afferents have not been demonstrated in the IML.

SP antagonist IV had effects different from the other three SP antagonists. Intrathecal injection: 1) increased baseline HR, 2) did not alter baseline MAP, and 3) did not significantly block the pressor and tachycardic responses to BMI applied to the VSMO (Figures 26,27). There is evidence for SP antagonist activity of this octa-peptide in peripheral bioassays [Mizrahi et al., 1982a,b, 1984]. This group reported that SP antagonist IV is a slightly more potent antagonist than SP antagonist I in its ability to inhibit SP-induced guinea pig ileum contractions. These findings apparently cannot be extrapolated to include potencies in the spinal cord. Binding studies with rat spinal cord membranes show that SP antagonist IV is about seven times less potent than the other three [Charlton and Helke, 1984a]. I attempted to duplicate this seven-fold potency difference in the i.t. preparation. Increasing the dose of antagonist IV by 50% (75 µg, the upper limits of its solubility in 7.5 µl)

did not block the cardiovascular effects evoked by BMI application to the VSMO. Since 5 µg doses of the SP antagonists I-III were effective, assuming equivalent bioavailability, SP antagonist IV was more than 15 times less potent (if indeed it had antagonist properties in the spinal cord). The reasons for the different potencies or antagonist properties in the periphery and the spinal cord are not clear. However, removal of the N-terminal tripeptide, the D-amino acid substitution at the number 4 position, and/or the D-Trp substitution at the number 10 position of the SP molecule significantly modifies its action on SP receptive neurons in the spinal cord which are involved with cardiovascular sympathetic function. Jones et al. [1983] and Morin-Surin et al. [1984] also reported the ineffectiveness of SP antagonist IV (and other SP antagonists) to block the actions of SP when administered iontophoretically into the CNS. The effects of SP antagonists I, II and IV injected into various CNS sites in vivo have been documented [Engberg et al., 1981; Lembeck et al., 1981; Piercey et al., 1981a; Akerman et al., 1982; Fuxe et al., 1982a; Loewy and Sawyer, 1982; Salt et al., 1982; Donnerer and Lembeck, 1983; Fasmer and Post, 1983; Stoppini et al., 1983; Yashpal et al., 1983], however this was the first in vivo use of SP antagonist III. In vitro studies with SP antagonist III in the perfused isolated spinal cord of neonatal rats [Yanagisawa, 1982], or various peripheral smooth muscle preparations from guinea pig, rat, and hamster [Rosell et al., 1983; Watson, 1983; Regoli et al., 1984d] showed this antagonist to be competitive for SP receptors and without agonist activity.

These results are in agreement with those of Loewy and Sawyer [1982]. In a similar study, they blocked the cardiovascular excitation by kainic acid applied to the VSMO with a SP antagonist injected i.t. My

results extended their findings in several ways: 1) The site of activation at the VSMO was more discretely localized i.e. to the intermediate area of the VSMO, and approximately 1/4 the area stimulated in Loewy and Sawyer's study. 2) Because multiple putative SP antagonists were used (three of which produced the same effects), this is evidence for some degree of specificity in this mimicry. 3) I showed also that a lower dose of SP antagonist (5 µg) injected i.t. could block cardiovascular excitation produced by stimulation of the VSMO neurons. 4) I ruled out the possibility of a non-specific neurotoxic action of i.t. injection of SP antagonist by following its time course of action, thus showing reversibility with time. 5) Finally, because I previously showed that VSMO excitation of the cardiovascular system with topical application of BMI in the rat is mediated by the peripheral sympathetic nervous system, the effects of SP antagonists in the spinal cord were a measure of central sympathetic involvement.

These data suggest that the influence of SP on sympathetic chronotropic tone is complex. Baseline HR was not changed by i.t. SP antagonist I, II or III (Figure 25), however the increases in HR produced by BMI at the VSMO were significantly decreased (Figure 25). There may be several other descending pathways that are important in providing an excitatory influence on HR. If SP transmission is interrupted in the spinal cord, its effects may not have been seen because of the formidable contribution of other neurotransmitters. However, when the SP influence is "amplified" by blocking GABA transmission at the VSMO, its role in sympathetic-mediated increases in HR are expressed. These findings could also imply that spinal cord SP is involved only in phasic HR responses. Indeed, increased blood flow responses to bilateral carotid artery occlusion were reduced by

topical application of GABA [Wennergren and Oberg, 1980]. and vasodepressor responses to increased pressure in the carotid sinus area were reduced by application of GABA antagonists [Yamada et al., 1984] to the S area of the VSMO in cats.

While recent in vivo studies with SP antagonists injected into the CNS support the specificity of these compounds for SP receptors [Engberg et al., 1981; Lembeck et al., 1981; Piercey et al., 1981a; Akerman et al., 1982, Hokfelt et al.,1981] showed immunohistochemical evidence for a neurotoxic action of a large dose of a SP antagonist microinjected into the rat brain. Neurotoxicity was not the nature of the antagonism seen in this study because the effects were reversible with time. Normal responses to BMI applied to the VSMO gradually returned in one to two hours (Figures 29,30).

I also attempted to verify the specificity of the SP antagonists' interactions by injecting a SP monoclonal antibody i.t. in three rats. This technique of immunoneutralization of a peptide has been used successfully as an alternative to receptor blockade in the CNS. An antiserum to SP administered into the substantia nigra by a push-pull cannula superfusion system antagonized the action of endogenous SP to stimulate dopamine release in the caudate nucleus. This effect was seen 10 minutes after the start of the infusion and was dose-dependent (1:10-10,000 v/v) [Cheramy et al., 1978]. In my experiments, i.t. injection of a SP monoclonal antibody (undiluted) with demonstrated effectiveness in radioimmunoassay and immunohistochemical procedures, did not block the bulbospinal excitatory activity even after two hours of observation, probably suggesting that the antibody did not penetrate the spinal cord.

Thus, the role of SP in spinal sympathetic pathways to the

cardiovascular system were deduced largely by the SP antagonist studies. I then began to study the effects of SP agonists in the spinal cord in order to verify the presumed sympathoexcitatory role of SP on the cardiovascular system. I wanted to study receptor interactions in the spinal cord physiologically, and to verify that the site of action of the SP antagonists was at SP receptors by reversing their effects with the similar administration of SP.

I attempted to assess the cardiovascular effects of SP in doses from 6 fmol to 60 nmol i.t. The expected dose-dependent pressor effects did not occur. Intrathecal doses high enough to evoke cardiovascular responses (greater than 6 pmol) apparently leaked into the periphery, because the responses were indistinguishable from those produced by equal doses given i.v. These were depressor and tachycardic responses and are consistent with the responses reported by Bury and Mashford [1977]. This group noted qualitatively similar effects to i.v. administration of SP in rats, rabbits, and dogs. Close femoral arterial injection of very small doses of SP (59 fmol/kg in dogs) evoked hypotensive effects and suggested an apparent direct action on vascular smooth muscle. The tachycardia was probably reflex mediated because it occurred concurrently with with the hypotension (my results and also those of Bury and Mashford) and SP (up to 3 nmol) had no effect on heart rate in a spontaneously beating isolated guinea pig heart preparation. In my experiments, doses 6 pmol or less had no appreciable effects on MAP or HR. This was most likely the result of rapid metabolic conversion of SP to inactive fragments. Indeed, the results of *in vivo* and *in vitro* studies indicate that SP is subject to degradation at multiple sites by different endogenous endopeptidases [Kato *et al.*, 1978; Heymann and Mentlein, 1978; Blumberg *et al.*, 1980; Lee *et*

al., 1981; Lockridge, 1982; Conlon and Sheehan, 1983; Cascieri et al., 1984; Skidgel et al., 1984], and the rapidity of this degradation has hampered the use of SP in in vivo experiments when injected into the CNS [Waldmeier et al., 1978]. The resultant difficulties in assessing the roles of SP in the CNS can therefore be overcome by either infusing SP [Waldmeier et al., 1978; Eison et al., 1982a,b], or by injecting stable active analogues of SP [Eison et al., 1982a,b].

Recently, a SP receptor agonist [pGlu^5, MePhe^8, MeGly^9]-SP(5-11) (DiME-SP) was developed and found to be resistant to enzymatic degradation because susceptible sites of attack are protected by methylated amino acids [Sandberg et al., 1981; Lee et al., 1981]. DiME-SP has 0.02 - 0.1 the potency of SP when tested in a variety of peripheral bioassays, 0.35 relative binding potency in rat brain membranes [Sandberg et al., 1981], and its effects appear to be related solely to actions at SP receptors [Hanley, 1983]. The binding kinetics of DiME-SP (and SP antagonist III) were also determined in rat spinal cord membranes [Keeler et al., 1984b]. [^{125}I]Bolton-Hunter-SP, a SP conjugate known to bind to high affinity SP receptors in a satuable manner [Charlton and Helke, 1984a], was dose-dependently inhibited by SP (IC_{50} 0.2 nM), DiME-SP (IC_{50} 1.5 μM), and SP antagonist III (IC_{50} 0.8 μM). DiME-SP has been compared with SP for its actions in the CNS. When equal doses of SP or DiME-SP were microinfused into the ventral tegmental area of rats, DiME-SP was as potent as SP in inducing (but longer lasting) qualitatively similar behavioral effects [Eison et al., 1982a]. In addition, DiME-SP produced some effects that were qualitatively dissimilar to SP. Unmetabolized tritiated analogue of DiME-SP was recovered at sites distant from the local injection site, indicating that diffusion of the stable analogue probably occurred [Eison

et al. 1982b].

Because of the problems with i.t. administration of SP, I used DiME-SP as a pharmacologic tool to further investigate the role of SP in spinal sympathetic pathways. Based on the data that implicated SP in an excitatory role to the cardiovascular system from my SP antagonist studies as well as the results of Loewy and Sawyer [1982] and Takano et al. [1984a], the results of these studies suggested that DiME-SP mimicked SP's actions in the spinal cord. Increasing doses of DiME-SP injected i.t. produced corresponding increases in MAP and HR that plateaued at 10-33 nmol (Figure 33).

The cardiovascular effects of DiME-SP in the spinal cord were mediated by the sympathetic nervous system. The excitatory nature of these responses is supported by iontophoretic studies that show SP in the IML excites antidromically identified preganglionic neurons [Gilbey et al., 1983; Backman and Henry, 1984]. The DiME-SP induced increases in MAP were accompanied by increases in plasma epinephrine and norepinephrine. Both responses were effectively abolished by ganglionic blockade with pentolinium (Figures 36,37). A tonic SP containing spinal sympathetic pathway from the VSMO to the peripheral vasculature is further suggested since disinhibition of a pressor pathway at the intermediate area of the VSMO with BMI was blocked by pentolinium i.v. (Figure 16) and SP antagonists I-III i.t. (Figure 26). Blockade by SP antagonist III was reversible with time (Figure 29) or by concurrent administration of DiME-SP i.t. (Figure 34). Attenuation of the DiME-SP-induced tachycardia by i.v. propranolol (Figure 36) and pentolinium (not shown) indicates that these effects effects are also mediated by the sympathetic nervous system. I was unable, however, to fully evaluate the relationship between the SP-containing

sympathetic pathway originating at the VSMO and its influence on chronotropic activity. Intrathecal administration of SP antagonist III alone did not change HR (Figure 25) but could block the BMI-induced (at the VSMO) tachycardia (Figure 27), indicating perhaps a phasic role of spinal SP on HR. When DiME-SP administration immediately preceded the SP antagonist i.t., a large increase in HR (to 474 bpm) was evoked, so that further sympathetic excitation by BMI at the VSMO was not possible.

Several lines of evidence support the idea that the DiME-SP-induced cardiovascular responses were mediated by SP receptors: 1) Pressor responses to kainic acid applied to the VSMO were correlated with increased SP in spinal cord CSF perfusates [Takano et al., 1984a]. 2) Pressor responses were also evoked by i.t. DiME-SP; a peptide that has been characterized as a SP receptor agonist in vitro and in vivo in rat brain [Sandberg et al., 1981; Eison et al., 1982a,b] and in rat spinal cord membranes [Keeler et al., 1984b]. 3) DiME-SP and SP antagonist III appear to be working through common receptors since DiME-SP antagonizes the depressor response to SP antagonist III and also antagonizes the SP antagonist III blockade of BMI-induced activation (at the VSMO) of pressor pathways (Figure 34). Furthermore, DiME-SP and SP antagonist III have similar affinities for SP receptors in spinal cord membrane preparations (IC_{50}'s of 1.5 and 0.8 μM, respectively) [Keeler et al., 1984b] and was reflected in similar efficaceous doses used in the in vivo experiments. Recently, Jensen et al. [1984a,b] reported that SP antagonist III also competitively inhibited bombesin-stimulated release of amylase from dispersed pancreatic acini. However, DiME-SP and SP antagonist III bound to SP receptors in rat spinal cord membranes, while bombesin did not interact with SP binding sites in the same system [Keeler et al., 1984b]. This suggested that DiME-SP and SP

antagonist III interact with receptors that are different from those for bombesin in the spinal cord.

Although the precise site of action of intrathecally administered drugs is not known, the likely site of the cardiovascular effects of DiME-SP was the IML. That DiME-SP interacted specifically with SP receptors and mimicked the expected cardiovascular effects of SP in the IML is consistent with the hypothesis that a SP-containing pathway from the VSMO to the IML mediates sympathoexcitatory information to the cardiovascular system.

Whereas an anatomic substrate for this pathway has been described [Helke et al., 1982], I cannot rule out the possibility that the cardiovascular effects produced by SP antagonists and DiME-SP were the result of interactions with SP receptors in a multisynaptic bulbospinal pathway. Indeed, inter- and intrasegmental SP-containing spinal neurons have been identified [Senba et al., 1981; Davis et al., 1983; and Davis and Cabot, 1984].

In summary, the pharmacologic evidence reported here supports the neuroanatomical, neurochemical, and electrophysiologic evidence for a role of spinal cord SP in cardiovascular regulation in the rat. These studies provide evidence that the excitatory cardiovascular effects evoked by the stimulation of cell bodies at the VSMO are due largely to SP transmission in the spinal cord, and these effects are mediated by the sympathetic nervous system. Needless to say, there are numerous experiments that could be done to further clarify this role and I propose a few experiments that would enhance my findings.

I tried to verify the specificity of the SP interaction in the spinal cord with the pharmacologic tools available, however further

testing should be done when more SP agonist analogues become available. Alternatively, one could superfuse SP i.t. to verify the cardiovascular responses produced by DiME-SP.

Electrophysiological experiments could help to extend my findings. DiME-SP, SP antagonists, and SP antibodies could be microinjected into antidromically identified sympathetic neurons in the IML while recording sympathetic nerve activity. The results from these experiments would more discretely localize the site of action of the cardiovascular effects I observed by i.t. administration of drugs. One could similarly evaluate the presumed origin of the SP bulbospinal pathway at the VSMO. A VSMO cell that was antidromically activated from the IML, which in turn was antidromically activated by sympathetic preganglionic nerve stimulation, would be intracellularly labeled. Immunohistochemical processing of the medulla would determine if the intracellularly labeled VSMO cell contained SP.

The origin of the SP-containing bulbospinal pathway could be more discretely localized anatomically and pharmacologically. A retrograde tracer could be microinjected into the IML. After a suitable survival time, rats would be killed and their brains processed for visualization of labeled cell bodies in the PGCL/VSMO, and also processed for immunohistochemical localization of SP. Double labeled cells would suggest a monosynaptic relationship between SP-containing neurons of the PGCL/VSMO and sympathetic preganglionic neurons. In order to verify the pathway's cardiovascular function, a retrogradely transported neurotoxin could be microinjected into the IML. SP antagonists injected i.t. should not cause depressor responses and even the baseline blood pressure should be below normal since this manipulation would eliminate the tonically active excitatory pathway. In addition, topical application of GABA of BMI to the

VSMO should be ineffective in changing the blood pressure and/or heart rate. At the end of the procedure, rats' brains would be processed for SP immunohistochemistry to verify loss of SP-containing neurons at the VSMO/PGCL.

My studies concerned the role of the VSMO/PGCL in maintenance of cardiovascular tone. Related questions include: 1) What is the VSMO/PGCL's role in cardiovascular reflexes? 2) Where does the tonically released GABA come from? 3) What are the functions of other neurotransmitters indigenous to the PGCL? 4) How do other PGCL efferent and afferent pathways contribute to the overall integrity of the cardiovascular system? These and other questions are currently being addressed by several laboratories and the answers will lay the foundation for clinical approaches to alleviating cardiovascular related pathologies.

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